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CYTOKINETICS

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To:	Kamrin MacKnight	**From:**	Roman Sakowicz
Fax:	415.397.8338	**Pages:**	(Including cover sheet)
Phone:		**Date:**	
Re:		**CC:**	

☐ **Urgent** ☐ **For Review** ☐ **Please Comment** ☐ **Please Reply** ☐ **Please Recycle**

Hi Kamrin,

The following pages contain my signed declaration. I will send you the oryginal by mail.

Roman

PATENT
Attorney Docket No. UCSD-04742

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Sakowicz and Goldstein		
Serial No.:	09/235,416	Group No.:	1645
Filed:	01/22/99	Examiner:	L. Li
Entitled:	**IDENTIFICATION AND EXPRESSION OF A NOVEL KINESIN MOTOR PROTEIN**		

DECLARATION OF ROMAN SAKOWICZ, Ph.D. UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 CFR 1.8(a)(1)(i)(A):

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on January 8, 2001.

By: _____
Marlene E. Carrejo

Sir:

1. I, Roman Sakowicz, am a co-inventor of the subject matter embodied in the above-identified patent application.

2. I am familiar with the Office Action from the Patent Office mailed September 7, 2000, in the above-named application.

3. In the Office Action, the Examiner rejects Claims 34, 36-39 and 41, under 35 U.S.C. §102(a), as being anticipated under Au-Young (WO 97/43413). I have reviewed WO 97/43413.

4. In this Office Action, the Examiner also rejects Claims 34-41, under 35 U.S.C. §103(a) as being obvious under Au-Young (WO 97/43413) combined with Foulkes *et al.* (U.S. Patent No. 5,580,772). In addition to Wo 97/43413, I have reviewed U.S. Patent No. 5,580,772.

5. The WO 97/43413 publication describes a human homolog of the yeast (*Saccharomyces cerevisiae*) PAC10 protein. The Examiner states that Au-Young teaches that the "PAC10 protein is a heavy chain of the kinesin family" (Office Action, page 5). Although this publication indicates that the Pac10 gene of *Saccharomyces cerevisiae* encodes the heavy

chain of members of the kinesin family, this statement has not been substantiated and there is evidence that this is not a correct statement.

6. For example, as shown in the BLAST search and sequence entry attached hereto at Tab 1, Pac10 has *no* homology to the kinesin motor domain. The kinesin motor domain is a defining signature of kinesin family members, as indicated in the present Specification (*See,* page 1, lines 23-31; page 9, lines 6-9; and page 22, lines 26-30, etc.). Thus, as there is no detectable homology ere is no evidence that the Pac10 protein is structurally related to kinesins, as claimed in the present application.

7. In addition, Pac10 is a small protein of only 180 amino acids. This protein is predicted to form coiled coils (*See e.g.,* Geissler *et al.,* EMBO J., 17:952-966 [1998], at page 953; attached hereto at Tab 2). As is known in the field, coiled coil regions sometimes result in low homology BLAST hits with proteins containing long stretches of coiled coils, such as myosins, kinesins and neurofilaments. In contrast kinesin minimal motor domains, as indicated by x-ray structural analyses, is a globular protein of more than 300 amino acids (*See e.g.,* Specification at page 1, lines 29-31; and page 9, lines 6-9, etc.).

8. Also, there is no literature evidence of which I am aware that indicates Pac10 has a motor function consistent with its being a kinesin. On the contrary, there is good evidence that Pac10 is involved in the formation of functional tubulin molecules.

9. In addition, as described in Geissler *et al.,* EMBO J., 17:952-966 [1998]; attached hereto at Tab 2), *GIM2,* which was identified as *PAC10,* is part of a protein complex that promotes formation of functional alpha and gamma tubulin. As discussed above, these *GIM* proteins are phylogenetically conserved coiled coil proteins. Deletion of the *GIM* genes causes microtubule defects. Indeed, all *gim* null strains were found to be super-sensitive to 2.5 μg/ml benomyl, an indicator of proteins associated with microtubule biogenesis. The reference postulates that ". . . the reduced level of α-tubulin in Δ*pac10* cells may explain why this gene becomes essential in the absence of the microtubule-binding protein Cin8p (Gieser *et al.,* 1997). Alternatively Pac10p may have additional motor protein-*related* functions" (*See,* page 960, *emphasis added*). Although there is no evidence presented to support this latter hypothesis, it is important to note that the authors do not suggest that Pac10p is a motor protein itself. Rather, the authors suggest the possibility that Pac10p functions affect the motor proteins. Indeed, there is ample evidence provided in this reference that would point away from any relationship between Pac10p and kinesin motor proteins, including the

structure, size, and other functions described in this reference itself (*e.g.*, the statements that the Gim proteins form a protein complex that promotes formation of functional α- and γ-tubulin, a function that is very different from the motor functions of the presently claimed invention.

10. Additional evidence that Pac10 is very different from the TL-γ protein of the presently claimed invention is provided in Alvarez *et al.* (Alvarez *et al.*, Genetics 149:857-864 [1998]; attached hereto at Tab 3). In this reference, the authors indicate that Pac10 is a protein that is involved in modulation of tubulin polypeptide ratios. Indeed, this reference clearly indicates that Pac10 lacks motor functions, as indicated on page 863:

> "*PAC10* originally was identified as a gene required for viability in the absence of the mitotic motor protein Cin8p (GEISER *et al.* 1997). However, like some of the other genes so identified, Pac10p does *not* appear to have motor functions, so its absence could act indirectly to exacerbate the sublethal consequences of a *CIN8* deletion." (Alvarez *et al.*, at page 863, *emphasis added*).

11. I know of no evidence in the scientific literature that Pac10 and the TL-γ of the presently claimed invention are the same. Nor is there any support for any relationship between these proteins based on homologies or structural similarities.

12. In addition, the assays of Foulkes *et al.* are directed towards identification of compounds for treatment of cardiovascular disease. The assay set forth within the Foulkes *et al.* Patent is a general protocol for high throughput testing. Thus, there is nothing in Foulkes *et al.* Patent that would indicate their high throughput screening methods would be suitable to detect TL-γ modulators, as claimed in the present application. Indeed, as the protocols of the Foulkes *et al.* Patent do not involve such elements as plus end-directed microtubule motor activity among other factors, there is no indication that the assays of Foulkes *et al.* would work as a method to screen for modulators of TL-γ.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Dated: 01/08/2001 Signed: _____
ROMAN SAKOWICZ, Ph.D.

LOCUS S59741 199 aa PLN 20-JUN-2000
DEFINITION PAC10 protein - yeast (Saccharomyces cerevisiae).
ACCESSION S59741
PID g2132376
VERSION S59741 GI:2132376
DBSOURCE pir: locus S59741;
summary: #length 199 #molecular-weight 23115 #checksum 6623;
genetic: #gene SGD:PAC10 ##cross-references SGD:S0003310;
MIPS:YGR078c #map_position 7R;
superfamily: Caenorhabditis elegans hypothetical protein T06G6.9;
PIR dates: 13-Jan-1996 #sequence_revision 01-Mar-1996 #text_change
20-Jun-2000.
KEYWORDS .
SOURCE baker's yeast.
ORGANISM Saccharomyces cerevisiae
Eukaryota; Fungi; Ascomycota; Saccharomycetes; Saccharomycetales;
Saccharomycetaceae; Saccharomyces.
REFERENCE 1 (residues 1 to 199)
AUTHORS Geiser,J.R. and Hoyt,M.A.
TITLE Direct Submission
JOURNAL Submitted (??-JUN-1995) to the EMBL Data Library
REFERENCE 2 (residues 1 to 199)
AUTHORS Wedler,H., Scharfe,M., Wedler,E. and Wambutt,R.
TITLE Direct Submission
JOURNAL Submitted (??-MAY-1996) to the Protein Sequence Database
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/organism="Saccharomyces cerevisiae"
/db_xref="taxon:4932"
Protein 1..199
/product="PAC10 protein"
/note="protein G4579; protein YGR078c"
ORIGIN
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121 glwlgadvml eypideaiel lkkkladseq sltvstedve flrenittme vncarlynwd
181 vqrrqdlkqa qegtknlki
//

BLASTP 2.1.2 [Nov-13-2000]

Reference <http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?uid=9254694&form=6&db=m&Dopt=r>:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 978722125-16435-30786

Query=
(199 letters)

Database: nr
601,330 sequences; 190,136,905 total letters

If you have any problems or questions with the results of this search please refer to the BLAST FAQs
<http://www.ncbi.nlm.nih.gov/blast/blast_FAQs.html>

Top of Form 1

Taxonomy reports <blast.cgi?RID=978722125-16435-30786&ALIGNMENT_VIEW=17>

Score
E
Sequences producing significant alignments:
(bits) Value

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Alignments

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Query: 14  GIPQAPFIENVNEIIKDPSD--FELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLEN
71
           GIP+A F+E+V+  +K P +   +    K  E+  KYKFM+ +     ++LK +IP+++
Sbjct: 24  GIPEAVFVEDVDSFMKQPGNETADTVLKKLDEQYQKYKFMELNLAQKKRRLKGQIPEIKQ
83

Query: 72  TLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLE
131
           TL+I + ++       ES   +   + L D LY KA V  P D    KV LWLGA+VMLE
Sbjct: 84  TLEILKYMQKKK----ESTNSMETRFLLADNLYCKASVP-PTD----KVCLWLGANVMLE
134

Query: 132 YPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQ
191
           Y IDEA  LL+K L+ + ++L    ED++FLR+  TT EVN AR+YNWDV+RR
Sbjct: 135 YDIDEAQALLEKNLSTATKNLDSLEEDLDFLRDQFTTTEVNMARVYNWDVKRR----NKD
190

Query: 192 EGTKN 196
           + TKN
Sbjct: 191 DSTKN 195
>sp|Q10143|YAS7_SCHPO
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01723218&dopt=GenPept> HYPOTHETICAL 19.5 KD PROTEIN C3H8.07C
IN CHROMOSOME I
 pir||T38765
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07493166&dopt=GenPept> probable VBP1/PAC10 family protein -
fission yeast
           (Schizosaccharomyces pombe)
 emb|CAA93164.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01177665&dopt=GenPept> (Z69086) putative VBP1/PAC10 family
protein [Schizosaccharomyces
           pombe]
          Length = 169

 Score =  127 bits (320), Expect = 7e-29
```

```
 Identities = 77/181 (42%), Positives = 109/181 (59%), Gaps = 14/181
(7%)

Query: 8   TEKNARGIPQAPFIENVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIP
67
            +  N RGIP A F E   E+  + +   L   KFQE ++KYKFM+ S +  +  L  +IP
Sbjct: 2   SSSNPRGIPPAQFFE-FKELSMEEAQGHL--EKFQEAIAKYKFMETSVVRRVASLDDKIP
58

Query: 68  DLENTLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGAD
127
            D+  TL+  Q L+    +GD       + Y+LNDTL  KA+V+  ++     V LWLGA+
Sbjct: 59  DIRKTLQSVQFLKER--QGDS----FTVTYELNDTLNAKAEVEAKDN-----VYLWLGAN
107

Query: 128 VMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDL
187
            VMLEY ++EA  LL +KL  +E++L    ED+EFLR  +TTMEVN AR+YN+ V  R+
Sbjct: 108 VMLEYTVEEAEALLTQKLNSAEETLKACKEDLEFLRAQVTTMEVNTARVYNYTVLLRKKT
167

Query: 188 K 188
           K
Sbjct: 168 K 168
>ref|XP_010231.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=11420139&dopt=GenPept> von Hippel-Lindau binding protein 1
[Homo sapiens]
          Length = 233

 Score =  126 bits (317), Expect = 2e-28
 Identities = 76/185 (41%), Positives = 111/185 (59%), Gaps = 15/185
(8%)

Query: 14  GIPQAPFIENVNEIIKDPSD--FELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLEN
71
            GIP+A F+E+V+  +K P +   +    K  E+  KYKFM+ +     ++LK +IP+++
Sbjct: 60  GIPEAVFVEDVDSFMKQPGNETADTVLKKLDEQYQKYKFMELNLAQKKRRLKGQIPEIKQ
119

Query: 72  TLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLE
131
            TL+I + ++       ES   +   + L D LY KA V  P D    K+ LWLGA+VMLE
Sbjct: 120 TLEILKYMQKKK----ESTNSMETRFLLADNLYCKASVP-PTD----KMCLWLGANVMLE
170

Query: 132 YPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQ
191
            Y IDEA  LL+K L+ + ++L    ED++FLR+  TT EVN AR+YNWDV+RR
Sbjct: 171 YDIDEAQALLEKNLSTATKNLDSLEEDLDFLRDQFTTTEVNMARVYNWDVKRR----NKD
226

Query: 192 EGTKN 196
           + TKN
Sbjct: 227 DSTKN 231
>gb|AAC50617.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
```

n&list_uids=01465751&dopt=GenPept> (U56833) VHL binding protein-1 [Homo sapiens]
Length = 166

Score = 116 bits (290), Expect = 2e-25
Identities = 72/177 (40%), Positives = 104/177 (58%), Gaps = 15/177 (8%)

```
Query: 22  ENVNEIIKDPSD--FELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSL 79
           E+V+  +K P +   +    K  E+  KYKFM+ +     ++LK +IP+++ TL+I + +
Sbjct: 1   EDVDSFMKQPGNETADTVLKKLDEQYQKYKFMELNLAQKKRRLKGQIPEIKQTLEILKYM 60

Query: 80  RNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIE 139
           +       ES   +   + L D LY KA V  P D    KV LWLGA+VMLEY IDEA
Sbjct: 61  QKKK----ESTNSMETRFLLADNLYCKASVP-PTD----KVCLWLGANVMLEYDIDEAQA 111

Query: 140 LLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQEGTKN 196
           LL+K L+ + ++L    ED++FLR+  TT EVN AR+YNWDV+RR       + TKN
Sbjct: 112 LLEKNLSTATKNLDSLEEDLDFLRDQFTTTEVNMARVYNWDVKRR----NKDDSTKN 164
```

>gb|AAF54630.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=07299441&dopt=GenPept> (AE003691) CG6719 gene product [Drosophila melanogaster]
Length = 194

Score = 113 bits (282), Expect = 2e-24
Identities = 67/179 (37%), Positives = 102/179 (56%), Gaps = 14/179 (7%)

```
Query: 9   EKNARGIPQAPFIENVNEIIKDPSD--FELCFNKFQERLSKYKFMQESKLATIKQLKTRI 66
           +K   GIP+A F+E ++  +  P +   E    +  E+  KY+FM  +  A  ++LK++I
Sbjct: 17  QKTFAGIPEAVFLEEIDTFMSQPENENCEKVLQRLDEQHGKYRFMACNLEARRRKLKSQI 76

Query: 67  PDLENTLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGA 126
           PDLE +L++   LR   +E +          + L+D ++ K  V  P  +    V LWLGA
Sbjct: 77  PDLERSLEMVNVLRKEDEERETQ-------FLLSDQVFIKTLV--PPTKT---VYLWLGA 124

Query: 127 DVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQ 185
            VMLEYP+DEA  LL + +  +  +L     D +FLR+ ITT EVN AR+YNW V++RQ
Sbjct: 125 SVMLEYPLDEAEALLNQNITSAVGNLKSVEHDQDFLRDQITTTEVNMARVYNWGVKKRQ 183
```

>ref|NP_035822.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=06755959&dopt=GenPept> von Hippel-Lindau binding protein 1 [Mus musculus]
sp|Q15765|VBP1_HUMAN
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=03924643&dopt=GenPept> VON HIPPEL-LINDAU BINDING PROTEIN 1

(VHL BINDING PROTEIN-1) (VBP-1)
(HIBBJ46)
gb|AAC23907.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=02738244&dopt=GenPept> (U96759) von Hippel-Lindau binding protein [Homo sapiens]
gb|AAC23908.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=02738246&dopt=GenPept> (U96760) von Hippel-Lindau binding protein homolog [Mus musculus]
Length = 160

Score = 112 bits (280), Expect = 3e-24
Identities = 68/157 (43%), Positives = 95/157 (60%), Gaps = 13/157 (8%)

```
Query: 40  KFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLRNHSDEGDESDEPILLHYQL 99
           K  E+  KYKFM+ +     ++LK +IP+++ TL+I + ++       ES   +   + L
Sbjct: 15  KLDEQYQKYKFMELNLAQKKRRLKGQIPEIKQTLEILKYMQKKK----ESTNSMETRFLL 70

Query: 100 NDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDV 159
            D LY KA V  P D    KV LWLGA+VMLEY IDEA  LL+K L+ + ++L    ED+
Sbjct: 71  ADNLYCKASVP-PTD----KVCLWLGANVMLEYDIDEAQALLEKNLSTATKNLDSLEEDL 125

Query: 160 EFLRENITTMEVNCARLYNWDVQRRQDLKQAQEGTKN 196
           +FLR+  TT EVN AR+YNWDV+RR       + TKN
Sbjct: 126 DFLRDQFTTTEVNMARVYNWDVKRR----NKDDSTKN 158
```

>dbj|BAB10764.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=10177617&dopt=GenPept> (AB023033) von Hippel-Lindau binding protein (VHL binding protein;
VBP) like [Arabidopsis thaliana]
Length = 195

Score = 112 bits (280), Expect = 3e-24
Identities = 66/177 (37%), Positives = 106/177 (59%), Gaps = 16/177 (9%)

```
Query: 13  RGIPQAPFIENVNEIIK----DPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPD 68
           RGIP A FI++V   +     DP+   L F+  QERL +YK ++   LA  + L+ +IPD
Sbjct: 17  RGIPAAKFIQDVETYLSQSGLDPNS-ALAFH--QERLQQYKVVEMKLLAQQRDLQAKIPD 73

Query: 69  LENTLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADV 128
           +E  L++  +L      G    E +L  +++++ +Y++A ++  +      V LWLGA+V
Sbjct: 74  IEKCLEVVATLEAKKGTG----EALLADFEVSEGIYSRACIEDTDS-----VCLWLGANV 124

Query: 129 MLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQ 185
           MLEY  +EA  LLK  L +++ SL V   D++FLR+ +T  +V  AR+YNWDV +R+
Sbjct: 125 MLEYSCEEASALLKNNLENAKASLEVLVADLQFLRDQVTVTQVTIARVYNWDVHQRR 181
```

>sp|O18054|YFM9_CAEEL

<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=03915477&dopt=GenPept> HYPOTHETICAL 20.9 KD PROTEIN T06G6.9
IN CHROMOSOME I
 pir||T24621
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07507330&dopt=GenPept> hypothetical protein T06G6.9 -
Caenorhabditis elegans
 emb|CAB04707.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=03879580&dopt=GenPept> (Z81587) Similarity to Human VHL
binding protein-1 (TR:Q15765)~cDNA
           EST yk171c9.5 comes from this gene~cDNA EST yk146c6.3
           comes from this gene~cDNA EST yk486h8.3 comes from this
           gene [Caenorhabditis elegans]
          Length = 185

 Score = 103 bits (256), Expect = 2e-21
 Identities = 64/186 (34%), Positives = 103/186 (54%), Gaps = 10/186
(5%)

Query: 11 NARGIPQAPFIENVNE-IIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDL
69
           +ARGIP++ IE+V   + K+   E   +E+ KYK+++ S LA   ++ +IP+
Sbjct: 9   SARGIPKSELIEDVESWLTKEKLSIEEAEVVLREKYGKYKYVESSMLAQKVRMSEKIPEF
68

Query: 70 ENTLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVM
129
           EN+L I +L   DES E   L   L+D +YTKA V PE   KV +WLGA+VM
Sbjct: 69 ENSLSIIDTLIAKR-AADESFETTFL---LSDDVYTKATVQKPE-----KVSIWLGANVM
119

Query: 130 LEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQ
189
           +EY ++ A +LL K   ++ +   T ++ ++++ ITT EVN + + N+ V +R+
Sbjct: 120 VEYDLENARKLLDKNRGSVQKVVDELTNELSYIKDQITTTEVNMSHIVNFGVNKRRAALA
179

Query: 190 AQEGTK 195
              G K
Sbjct: 180 VNNGAK 185
>emb|CAB98114.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=09828025&dopt=GenPept> (AL160493) probable prefoldin subunit
3 [Leishmania major]
          Length = 207

 Score = 94.0 bits (232), Expect = 1e-18
 Identities = 54/164 (32%), Positives = 93/164 (55%), Gaps = 7/164 (4%)

Query: 5   FNSTEKNARGIPQAPFIENVNEIIKDPSDF-ELCFNKFQERLSKYKFMQESKLATIKQLK
63
           F   + RGIP+ F+ENV E++K   D E   +F E+ SKYK +   + T   L+
Sbjct: 11 FKDDYVSPRGIPKVAFVENVAELVKSSGDSAETLLKRFSEQYSKYKLAEHRLIRTTANLE
70

Query: 64 TRIPDLENTLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLW

```
123
            +IPD++ TL+   + L+   S    +          +Y L ++++ +A+V +P+       V LW
Sbjct: 71  AKIPDIKKTLQTLEYLKK-SLVAENGGRGFTTNYGLTESVFCQAKV-LPQKT----VHLW
124

Query: 124 LGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENIT 167
           LGA+VM+EY  +EA +LL++ L   + ++L  + ED+ +L+E  T
Sbjct: 125 LGANVMVEYTFEEATQLLERNLKSATENLAATQEDLAWLQEQQT 168
>gb|AAF46750.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07291320&dopt=GenPept> (AE003454) CG15676 gene product
[Drosophila melanogaster]
          Length = 163

 Score = 48.9 bits (115), Expect = 4e-05
 Identities = 41/173 (23%), Positives = 84/173 (47%), Gaps = 18/173
(10%)

Query: 15  IPQAPFIENVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLAT-----IKQLKTRIPDL
69
           IP+A  +++V   I  P  +          ++ +  ++Q S+LA      +  + TR+
Sbjct: 3   IPEAKLVDDVVSYIAKPEFYSTVPAAL--KMQRLFYVQYSELAAKLETDLTAVLTRLEAA
60

Query: 70  ENTLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVM
129
           +N L++   +R   D  D+    ++   Q+   ++    V IP  +   KV L +GA +
Sbjct: 61  KNNLEL---VRRFIDNPDKEVHSLV---QIAQGVFR--WVSIPPVQ---KVTLQVGASLQ
109

Query: 130 LEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQ 182
           +E+ + EA E +KK +    +       D+++L++ + T+E+N A LY  +V+
Sbjct: 110 MEFELSEAEEFIKKDITSLVKQQLQHEHDIDYLQDQVNTIEMNLAVLYKHEVE 162
>pir||S70633
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=02136815&dopt=GenPept> serine/threonine-specific protein
kinase (EC 2.7.1.-), Rho-associated
           - bovine
 gb|AAC48567.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01326078&dopt=GenPept> (U36909) Rho-associated kinase [Bos
taurus]
          Length = 1388

 Score = 37.7 bits (86), Expect = 0.098
 Identities = 41/173 (23%), Positives = 68/173 (38%), Gaps = 12/173 (6%)

Query: 26   EIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLRNHSDE
85
           E+ K+  D +    + Q++L   ++        +++LK    + E   K+C+ L+    E
Sbjct: 847  ELRKERQDADGQMKELQDQLEAEQYFSTLYKTQVRELKE---ECEEKTKLCKELQQKKQE
903

Query: 86   GDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKKL
145
               DE   L  QL  TL       +    A+ +        +M E  I       K+ +
Sbjct: 904  --LQDERDSLAAQLEITLTKADSEQLARSIAEEQYSDLEKEKIMKELEI-------KEMM
```

954

```
Query: 146  ADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQEGTKNLK 198
            A  +Q LT     +  L E   T+  + A L N   +    LK+AQE    LK
Sbjct: 955  ARHKQELTEKDATIASLEETNRTLTSDVANLANEKEELNNKLKEAQEQLSRLK 1007
>emb|CAB38183.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=04468708&dopt=GenPept> (AJ005019) intermediate filament
protein IF2 [Sagitta elegans]
          Length = 526

 Score = 37.0 bits (84), Expect = 0.17
 Identities = 38/168 (22%), Positives = 77/168 (45%), Gaps = 22/168
(13%)

Query: 29   KDPSDFELCFNKFQERLSKYKFMQESKL---ATIKQLKTRIPDLENTLKICQSLRNHSDE
85
            ++  D +   ++F   + + +F++       + I  LK +   LE TLK       +  E
Sbjct: 98   REKRDLQELNDRFASYIERVRFLEADNKRLQSIIDVLKVKFEKLEETLK-----EMYEAE
152

Query: 86   GDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIE--LLKK
143
             D++ + I      ++T   KA+V++   R + ++     AD    Y  DEA E  + K
Sbjct: 153  LDQARKTI------DETTKAKAEVELKVARLEEEL-----ADYRRRYE-DEAREHAITKA
200

Query: 144  KLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQ 191
             +   E++++     ++FL +N+  +E   ARL     + ++DL  A+
Sbjct: 201  NIPKLEKAISERDAQIDFLTKNVDALERELARLKGEIARLQRDLSDAK 248
>pir||S72375
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07474137&dopt=GenPept> surface exclusion protein sep1
precursor - Enterococcus faecalis
           plasmid PD1
 emb|CAA65662.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01272653&dopt=GenPept> (X96976) surface exclusion protein
[Enterococcus faecalis]
          Length = 843

 Score = 35.4 bits (80), Expect = 0.49
 Identities = 35/180 (19%), Positives = 80/180 (44%), Gaps = 7/180 (3%)

Query: 23   NVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLRNH
82
            N  E ++  +  E    + +++L+    + +++ A +++ KT++ D +   +    +
Sbjct: 600  NHEEKVRQTAAAEKSLQQTKDQLATINELIQNRAAVLEKAKTKVADAQAIEQTTAKVLKE
659

Query: 83   SDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDE----AI
138
              E   ++E  L   Q    +   A+  + + +A LKV     + +    P  E    A+
Sbjct: 660  KQEAQRAEEEALKSLQ---EVLAIAKETLGKKQAQLKVSEQALSRLENAQPNYEKTVKAL
716

Query: 139  ELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQEGTKNLK
```

```
198
           E  KK L  +E++ T+S + +E L+E      +  A+        + +L+Q Q   ++L+
Sbjct: 717 EKAKKTLVHAEEAYTISLKSLEELKEQQAVATLAYAQAQEDLSNAKLELQQYQGVLRDLE
776
>ref|NP_011222.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=06321144&dopt=GenPept> SMC chromosomal ATPase family member;
Smc2p [Saccharomyces
           cerevisiae]
 sp|P38989|SMC2_YEAST
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=00730753&dopt=GenPept> CHROMOSOME SEGREGATION PROTEIN SMC2
(DA-BOX PROTEIN SMC2)
 pir||A56157
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01362470&dopt=GenPept> chromosome segregation protein SMC2 -
yeast (Saccharomyces
           cerevisiae)
 gb|AAA17416.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=00468040&dopt=GenPept> (U05820) Smc2p [Saccharomyces
cerevisiae]
 dbj|BAA09270.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=00836786&dopt=GenPept> (D50617) chromosome segregation
protein SMC2p [Saccharomyces
           cerevisiae]
 dbj|BAA08042.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=00893426&dopt=GenPept> (D44602) DA-box protein Smc2p
[Saccharomyces cerevisiae]
          Length = 1170

 Score = 34.7 bits (78), Expect = 0.83
 Identities = 21/65 (32%), Positives = 35/65 (53%), Gaps = 6/65 (9%)

Query: 138 IELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARL------YNWDVQRRQDLKQAQ
191
           +ELLKK+L   E  L  +T+D E   +++   +  C +L      Y +D  R +DLKQ +
Sbjct: 424 MELLKKELLTIEPKLKEATKDNELNVKHVKQCQETCDKLRARLVEYGFDPSRIKDLKQRE
483

Query: 192 EGTKN 196
           +  K+
Sbjct: 484 DKLKS 488
>gb|AAC67249.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=03435186&dopt=GenPept> (AF067175) HyaE [Pasteurella
multocida]
          Length = 622

 Score = 34.7 bits (78), Expect = 0.83
 Identities = 22/69 (31%), Positives = 38/69 (54%), Gaps = 5/69 (7%)

Query: 134 IDEAIELLKKKLADSEQSLTVSTEDVEFLR----ENITTMEVNCARLYNWDVQRRQDLKQ
189
           IDEA  L KK L+D E++L    +++E L+    ENI+  E +     +  +R+Q+L+Q
```

```
Sbjct: 395 IDEAYHLTKKTLSDKEKALKTHQDEIEALKIIFNENISVQE-DMQEKFQETNKRKQELEQ
453

Query: 190 AQEGTKNLK 198
             +   + K
Sbjct: 454 ELKAISDKK 462
>gb|AAD52842.1|AF134172_1
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=05817598&dopt=GenPept> (AF134172) myosin heavy chain [Pecten
maximus]
          Length = 1940

 Score = 33.9 bits (76), Expect = 1.4
 Identities = 49/202 (24%), Positives = 84/202 (41%), Gaps = 34/202
(16%)

Query: 22   ENVNEIIKDPSDFELCFNKFQERL-------SKYKFMQESKLATIKQLKTRIPDLENTLK
74
            E V ++I   +DFE    + +ERL       +  + +++        LK  I DLENTL+
Sbjct: 903  ERVEKLIMQKADFESQIKELEERLLDEEDAAADLEGIKKKMETDNSNLKKDIGDLENTLQ
962

Query: 75   ICQSLRNHSD--------EGDESDEPILLHYQLNDTL-----YTKAQVDIPEDRAD----
117
              +  + H D        E  + DE I    +    L      T   +   ED+ +
Sbjct: 963  KAEQDKAHKDNQISTLQGEMAQQDEHIGKLNKEKKALEEANKKTSESLQAEEDKCNHLNK
1022

Query: 118  LKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLY
177
            LK  L    D      +++ +E  KK   D E++     +D++  +EN+  +E    R
Sbjct: 1023 LKAKLEQALD-----ELEDNLEREKKVRGDVEKAKRKVEQDLKSTQENVEDLE-RVKREL
1076

Query: 178  NWDVQRRQDLKQAQEGTKNLKI 199
               +V+R    K+A+  T N K+
Sbjct: 1077 EENVRR----KEAEISTLNSKL 1094
>pir||T29095
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07512209&dopt=GenPept> cardiac muscle factor 1 - chicken
 gb|AAB36881.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01621107&dopt=GenPept> (U62026) cardiac muscle factor 1 CMF1
[Gallus gallus]
          Length = 1538

 Score = 33.9 bits (76), Expect = 1.4
 Identities = 42/167 (25%), Positives = 79/167 (47%), Gaps = 23/167
(13%)

Query: 35   ELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLRNHSDEGDESDEPIL
94
            E+     QE+++  +    S+   I+ LK+   +L N+LK        +   GD      L
Sbjct: 1265 EIQIEVLQEKVNSLESFISSQKLEIEHLKSTKEELNNSLK-----EANQTLGD------L
1313

Query: 95   LHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSL--
```

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152
            L  +  D + T  Q++  ++    +V LW+ +   +E    +  E+L+K+LAD E+ L
Sbjct: 1314 LKLKA-DNINTIVQLNKEKEFVQSEVQLWIKSCKQME----QEKEVLQKQLADCEELLKK
1368

Query: 153  -TVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQEGTKNLK 198
              +S  + E   EN  T E+   +L   ++Q   ++K  +E  +NL+
Sbjct: 1369 KDLSVSEKEGTDENAITEEI---KLELEELQEAVEVK-TREANENLE 1411
>ref|NP_015070.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=06325002&dopt=GenPept> Cik1p homolog; Vik1p [Saccharomyces
cerevisiae]
 pir||S61011
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=02132250&dopt=GenPept> hypothetical protein YPL253c - yeast
(Saccharomyces cerevisiae)
 emb|CAA91591.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01061236&dopt=GenPept> (Z67751) putative protein
[Saccharomyces cerevisiae]
 emb|CAA97978.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01370520&dopt=GenPept> (Z73609) ORF YPL253c [Saccharomyces
cerevisiae]
          Length = 647

 Score = 33.9 bits (76), Expect = 1.4
 Identities = 18/60 (30%), Positives = 32/60 (53%)

Query: 139 ELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQEGTKNLK
198
           E L K++ D ++    S +++  + ENI  +E+    L +  +Q  QDL+  Q   +NLK
Sbjct: 278 EKLHKEIMDIDRQAEYSEQNISEINENIKQLELANNPLISKSLQNSQDLEHLQNQMENLK
337
>pir||A75564
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07471324&dopt=GenPept> conserved hypothetical protein -
Deinococcus radiodurans  (strain
          R1)
 gb|AAF09665.1|AE001870_4
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=06457736&dopt=GenPept> (AE001870) conserved hypothetical
protein [Deinococcus radiodurans]
          Length = 1467

 Score = 33.9 bits (76), Expect = 1.4
 Identities = 19/61 (31%), Positives = 29/61 (47%)

Query: 134 IDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQEG
193
           + E    L  KL ++ Q  T + + V+ L E  T +    A+L    V     LK+AQ+G
Sbjct: 760 LSEGATTLSSKLKEAGQGSTAAVQGVQQLSEGATRLAAGTAQLSEGAVTLSSKLKEAQQG
819

Query: 194 T 194
           T
Sbjct: 820 T 820
```

>gb|AAF62394.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=07416982&dopt=GenPept> (AF183909) myosin heavy chain cardiac muscle specific isoform 1
[Argopecten irradians]
Length = 1243

Score = 33.5 bits (75), Expect = 1.8
Identities = 42/173 (24%), Positives = 72/173 (41%), Gaps = 29/173 (16%)

```
Query: 22  ENVNEIIKDPSDFELCFNKFQERL-------SKYKFMQESKLATIKQLKTRIPDLENTLK 74
           E V ++I   +DFE    + +ERL       +  + +++   A    LK  I DLENTL+
Sbjct: 181 ERVEKLIMQKADFESQIKELEERLLDEEDAAADLEGIKKKMEADNANLKKDIGDLENTLQ 240

Query: 75  ICQSLRNHSD--------EGDESDEPILLHYQLNDTL-----YTKAQVDIPEDRAD---- 117
             +  + H D        E  + DE I    +    L      T   +   ED+ +
Sbjct: 241 KAEQDKAHKDNQISTLQGEISQQDEHIGKLNKEKKALEEANKKTSDSLQAEEDKCNHLNK 300

Query: 118 LKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTME 170
           LK  L    D      +++ +E  KK   D E++     +D++  +EN+  +E
Sbjct: 301 LKAKLEQALD-----ELEDNLEREKKVRGDVEKAKRKVEQDLKSTQENVEDLE 348
>ref|NP_014550.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=06324481&dopt=GenPept> involved in sporulation; Spo21p [Saccharomyces cerevisiae]
 pir||S57378
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=01362354&dopt=GenPept> hypothetical protein YOL091w - yeast (Saccharomyces cerevisiae)
 emb|CAA58188.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=00600467&dopt=GenPept> (X83121) orf 00932 [Saccharomyces cerevisiae]
 emb|CAA99103.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=01419936&dopt=GenPept> (Z74833) ORF YOL091w [Saccharomyces cerevisiae]
Length = 609

Score = 33.5 bits (75), Expect = 1.8
Identities = 45/178 (25%), Positives = 79/178 (44%), Gaps = 31/178 (17%)

Query: 39  NKFQERLSKYKFMQESKL---ATIKQLKTRIPD--LENTLKICQSLRNHSDEGDESDEPI 93
           +K +E+ ++ K   +  L    T K  KT+  +  LEN  KI ++ +   +E    DE +
Sbjct: 291 SKLKEKEAQLKSQNDKILKLETTNKAYKTKYKEVSLENK-KIKEAFKELDNESYNHDEEL 349

Query: 94  LLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEA----------IELLKK 143
           L  Y+     YT+  +D    R + +  L +  +  L+ ++E                L K+
```

```
Sbjct: 350 LKKYK-----YTRETLD----RVNREQQLIIDQNEFLKKSVNELQNEVNATNFKFSLFKE
400

Query: 144 K---LADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQEGTKNLK
198
           K   LADS   L  ST+  E L EN+T     C  L    ++ +++++      KNL+
Sbjct: 401 KYAKLADSITELNTSTKKREALGENLT---FECNELKEICLKYKKNIENISNTNKNLQ
455
>gb|AAB19995.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=00236789&dopt=GenPept> myosin heavy chain=rod region
[Aequipecten irradians=scallops,
           Peptide Partial, 1104 aa]
          Length = 1104

 Score = 33.5 bits (75), Expect = 1.8
 Identities = 42/173 (24%), Positives = 72/173 (41%), Gaps = 29/173
(16%)

Query: 22  ENVNEIIKDPSDFELCFNKFQERL-------SKYKFMQESKLATIKQLKTRIPDLENTLK
74
           E V ++I   +DFE    + +ERL       +  + +++   A    LK  I DLENTL+
Sbjct: 66  ERVEKLIMQKADFESQIKELEERLLDEEDAAADLEGIKKKMEADNANLKKDIGDLENTLQ
125

Query: 75  ICQSLRNHSD--------EGDESDEPILLHYQLNDTL-----YTKAQVDIPEDRAD----
117
             +  + H D        E  + DE I    +    L      T   +   ED+ +
Sbjct: 126 KAEQDKAHKDNQISTLQGEISQQDEHIGKLNKEKKALEEANKKTSDSLQAEEDKCNHLNK
185

Query: 118 LKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTME 170
           LK  L    D      +++ +E  KK   D E++     +D++  +EN+  +E
Sbjct: 186 LKAKLEQALD-----ELEDNLEREKKVRGDVEKAKRKVEQDLKSTQENVEDLE 233
>gb|AAF62391.1|AF183909_1
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07416979&dopt=GenPept> (AF183909) myosin heavy chain
striated muscle specific isoform
           [Argopecten irradians]
          Length = 1219

 Score = 33.5 bits (75), Expect = 1.8
 Identities = 42/173 (24%), Positives = 72/173 (41%), Gaps = 29/173
(16%)

Query: 22  ENVNEIIKDPSDFELCFNKFQERL-------SKYKFMQESKLATIKQLKTRIPDLENTLK
74
           E V ++I   +DFE    + +ERL       +  + +++   A    LK  I DLENTL+
Sbjct: 181 ERVEKLIMQKADFESQIKELEERLLDEEDAAADLEGIKKKMEADNANLKKDIGDLENTLQ
240

Query: 75  ICQSLRNHSD--------EGDESDEPILLHYQLNDTL-----YTKAQVDIPEDRAD----
117
             +  + H D        E  + DE I    +    L      T   +   ED+ +
Sbjct: 241 KAEQDKAHKDNQISTLQGEISQQDEHIGKLNKEKKALEEANKKTSDSLQAEEDKCNHLNK
300
```

```
Query: 118 LKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTME 170
           LK  L    D      +++ +E  KK   D E++     +D++  +EN+  +E
Sbjct: 301 LKAKLEQALD-----ELEDNLEREKKVRGDVEKAKRKVEQDLKSTQENVEDLE 348
>gb|AAF62395.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07416983&dopt=GenPept> (AF183909) myosin heavy chain cardiac
muscle specific isoform 2
          [Argopecten irradians]
         Length = 1253

 Score = 33.5 bits (75), Expect = 1.8
 Identities = 42/173 (24%), Positives = 72/173 (41%), Gaps = 29/173
(16%)

Query: 22  ENVNEIIKDPSDFELCFNKFQERL-------SKYKFMQESKLATIKQLKTRIPDLENTLK
74
           E V ++I   +DFE    + +ERL       +  + +++   A    LK  I DLENTL+
Sbjct: 181 ERVEKLIMQKADFESQIKELEERLLDEEDAAADLEGIKKKMEADNANLKKDIGDLENTLQ
240

Query: 75  ICQSLRNHSD--------EGDESDEPILLHYQLNDTL-----YTKAQVDIPEDRAD----
117
             +  + H D        E  + DE I    +    L      T   +   ED+ +
Sbjct: 241 KAEQDKAHKDNQISTLQGEISQQDEHIGKLNKEKKALEEANKKTSDSLQAEEDKCNHLNK
300

Query: 118 LKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTME 170
           LK  L    D      +++ +E  KK   D E++     +D++  +EN+  +E
Sbjct: 301 LKAKLEQALD-----ELEDNLEREKKVRGDVEKAKRKVEQDLKSTQENVEDLE 348
>emb|CAB96710.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=08953662&dopt=GenPept> (AL360354) vir23, putative
transmembrane protein, low similarity to
          other VYIVD10 CDS [Plasmodium vivax]
         Length = 345

 Score = 33.5 bits (75), Expect = 1.8
 Identities = 32/148 (21%), Positives = 64/148 (42%), Gaps = 23/148
(15%)

Query: 43  ERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLRNHSDEGDESDEPILLHYQLNDT
102
           ++LS Y F +E     +K+LKT  PDL+   + CQ+    + + +  ++ I L  Q+ +
Sbjct: 20  KKLSLYDFYEELNKELVKELKTS-PDLQTKYQYCQA--KIASQAENGEKLINLCKQICNI
76

Query: 103 LYTKAQV-----DIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTE
157
           ++    +     D   ++A   +G WL  +VM                  + QSL ++
Sbjct: 77  IFNVHDILDKCNDKTGNKACNYMGYWLYYNVM--------------SLTNNQSLVLNFY
121

Query: 158 DVEFLRENITTMEVNCARLYNWDVQRRQ 185
           D  F+   +   + N   L N+ + + +
Sbjct: 122 DPIFMYTTVNKSKFNNCTLTNFKIDKNK 149
>gb|AAC46490.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
```

n&list_uids=00497653&dopt=GenPept> (U09782) myosin heavy chain
[Argopecten irradians]
 prf||2103335A
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01093399&dopt=GenPept> myosin:SUBUNIT=heavy chain
[Argopecten irradians]
          Length = 1951

 Score = 33.5 bits (75), Expect = 1.8
 Identities = 42/173 (24%), Positives = 72/173 (41%), Gaps = 29/173
(16%)

Query: 22   ENVNEIIKDPSDFELCFNKFQERL-------SKYKFMQESKLATIKQLKTRIPDLENTLK
74
             E V ++I   +DFE   + +ERL   + + +++   A   LK I DLENTL+
Sbjct: 903 ERVEKLIMQKADFESQIKELEERLLDEEDAAADLEGIKKKMEADNANLKKDIGDLENTLQ
962

Query: 75   ICQSLRNHSD--------EGDESDEPILLHYQLNDTL-----YTKAQVDIPEDRAD----
117
               + + H D   E + DE I   +   L   T   +   ED+ +
Sbjct: 963 KAEQDKAHKDNQISTLQGEISQQDEHIGKLNKEKKALEEANKKTSDSLQAEEDKCNHLNK
1022

Query: 118 LKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTME 170
             LK L   D   +++ +E KK   D E++   +D++ +EN+ +E
Sbjct: 1023 LKAKLEQALD-----ELEDNLEREKKVRGDVEKAKRKVEQDLKSTQENVEDLE 1070
>gb|AAF62392.1|AF183909_2
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07416980&dopt=GenPept> (AF183909) myosin heavy chain catch
(smooth) muscle specific
           isoform [Argopecten irradians]
          Length = 1229

 Score = 33.5 bits (75), Expect = 1.8
 Identities = 42/173 (24%), Positives = 72/173 (41%), Gaps = 29/173
(16%)

Query: 22 ENVNEIIKDPSDFELCFNKFQERL-------SKYKFMQESKLATIKQLKTRIPDLENTLK
74
             E V ++I   +DFE   + +ERL   + + +++   A   LK I DLENTL+
Sbjct: 181 ERVEKLIMQKADFESQIKELEERLLDEEDAAADLEGIKKKMEADNANLKKDIGDLENTLQ
240

Query: 75 ICQSLRNHSD--------EGDESDEPILLHYQLNDTL-----YTKAQVDIPEDRAD----
117
               + + H D   E + DE I   +   L   T   +   ED+ +
Sbjct: 241 KAEQDKAHKDNQISTLQGEISQQDEHIGKLNKEKKALEEANKKTSDSLQAEEDKCNHLNK
300

Query: 118 LKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTME 170
            LK L   D   +++ +E KK   D E++   +D++ +EN+ +E
Sbjct: 301 LKAKLEQALD-----ELEDNLEREKKVRGDVEKAKRKVEQDLKSTQENVEDLE 348
>sp|P24733|MYS_AEQIR
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=00127773&dopt=GenPept> MYOSIN HEAVY CHAIN, STRIATED MUSCLE
 pir||A40997

<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=00102700&dopt=GenPept> myosin heavy chain, striated adductor muscle - scallop (Aequipecten
irradians)
emb|CAA39247.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=00005612&dopt=GenPept> (X55714) myosin heavy chain [Argopecten irradians]
Length = 1938

Score = 33.5 bits (75), Expect = 1.8
Identities = 42/173 (24%), Positives = 72/173 (41%), Gaps = 29/173 (16%)

```
Query: 22   ENVNEIIKDPSDFELCFNKFQERL-------SKYKFMQESKLATIKQLKTRIPDLENTLK 74
             E V ++I   +DFE    + +ERL       +  + +++   A    LK  I DLENTL+
Sbjct: 900  ERVEKLIMQKADFESQIKELEERLLDEEDAAADLEGIKKKMEADNANLKKDIGDLENTLQ 959

Query: 75   ICQSLRNHSD--------EGDESDEPILLHYQLNDTL-----YTKAQVDIPEDRAD---- 117
               +  + H D        E  + DE I    +    L      T   +   ED+ +
Sbjct: 960  KAEQDKAHKDNQISTLQGEISQQDEHIGKLNKEKKALEEANKKTSDSLQAEEDKCNHLNK 1019

Query: 118  LKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRENITTME 170
             LK  L    D      +++ +E  KK   D E++     +D++  +EN+  +E
Sbjct: 1020 LKAKLEQALD-----ELEDNLEREKKVRGDVEKAKRKVEQDLKSTQENVEDLE 1067
>sp|Q10411|YD86_SCHPO
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=01723492&dopt=GenPept> HYPOTHETICAL 222.8 KD PROTEIN C1F3.06C IN CHROMOSOME I
pir||T38077
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=07490683&dopt=GenPept> hypothetical coiled-coil protein - fission yeast
(Schizosaccharomyces pombe)
emb|CAA94624.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=01256517&dopt=GenPept> (Z70690) hypothetical coiled-coil protein [Schizosaccharomyces
pombe]
Length = 1957

Score = 32.7 bits (73), Expect = 3.1
Identities = 40/174 (22%), Positives = 71/174 (39%), Gaps = 20/174 (11%)

Query: 25   NEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLRNHSD 84
             NE+ +  +  +   N FQE+L+K          ++ QLK    +  +     + L
Sbjct: 570  NELSESKNSLQTLCNAFQEKLAK----------SVMQLKENEQNFSSLDTSFKKLNESHQ 619

Query: 85   EGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKK 144
```

```
           E + + + I    QL DT     Q+ +     + K          + +   D   +LLK
Sbjct: 620 ELENNHQTITK--QLKDTSSKLQQLQLERANFEQK------ESTLSDENNDLRTKLLK--
669

Query: 145 LADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQEGTKNLK 198
           L +S +SL    EDV+ L +NI T++ +  +         + + K  +E   NLK
Sbjct: 670 LEESNKSLIKKQEDVDSLEKNIQTLKEDLRKSEEALRFSKLEAKNLREVIDNLK 723
>sp|Q92376|KLP1_SCHPO
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=03915752&dopt=GenPept> KINESIN-LIKE PROTEIN 1
 pir||T38749
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07492128&dopt=GenPept> kinesin-like protein 1 - fission
yeast (Schizosaccharomyces pombe)
 emb|CAB16597.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=02662026&dopt=GenPept> (Z99296) kinesin-like protein 1;Kar3
subfamily [Schizosaccharomyces
           pombe]
          Length = 832

 Score = 32.7 bits (73), Expect = 3.1
 Identities = 39/165 (23%), Positives = 76/165 (45%), Gaps = 18/165
(10%)

Query: 40  KFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLRNHSDEGDESDEPI-LLHYQ
98
           K QE   +   ++     T+K+LK RI +LE  +K  +S +++S++  E +E I  L  +
Sbjct: 331 KQQEENDRISHIEYENDLTVKKLKRRISELEMAVKEYESEKSYSEK--EYEEKISSLRIE
388

Query: 99  LNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTED
158
           L D L   A++D+  ++         L  +    +   E +E L K +A  +     + ++
Sbjct: 389 LEDKL---AEIDMLRNK--------LLKEEHKHHSTSEKLEELSKYVASIQDKERNNGQN
437

Query: 159 VEFLRENITTMEVNCARLYN----WDVQRRQDLKQAQEGTKNLKI 199
              L+  I  +E     +YN     ++ RR+     QE   N+++
Sbjct: 438 ALELQARIQQLERRNEDMYNKLLAEEIIRRKLHNDIQELKGNIRV 482
>pir||T29145
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07504365&dopt=GenPept> hypothetical protein F56A3.4 -
Caenorhabditis elegans
 gb|AAB37802.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01707041&dopt=GenPept> (U80446) coded for by C. elegans cDNA
yk7g12.3; coded for by C.
           elegans cDNA yk25e3.3; coded for by C. elegans cDNA
           yk28f12.3; coded for by C. elegans cDNA yk1b1.3; coded
           for by C. elegans cDNA yk155d11.3; coded for by C.
           elegans cDNA yk182d5.3; coded >
          Length = 1199

 Score = 32.3 bits (72), Expect = 4.1
 Identities = 16/64 (25%), Positives = 34/64 (53%)
```

```
Query: 22  ENVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLRN
81
           + V E++ D  D +L     QE++ +YK M+E  LA   +++T   +    +K  ++ ++
Sbjct: 99  KRVKEVMDDYVDLKLGQENVQEKMEQYKLMEEDLLAMQSRIETSEDNFARQMKEFEAQKH
158

Query: 82  HSDE 85
             +E
Sbjct: 159 AMEE 162
>pir||T23130
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07508648&dopt=GenPept> hypothetical protein T28B8.4 -
Caenorhabditis elegans
 emb|CAA15925.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=03878065&dopt=GenPept> (AL021066) Similarity to Human mRNA
product KIAA0077 (TR:Q14997)~cDNA
            EST yk243h8.5 comes from this gene~cDNA EST yk243h8.3
            comes from this gene~cDNA EST yk359h4.5 comes from this
            gene [Caenorhabditis elegans]
 emb|CAB03445.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=03880318&dopt=GenPept> (Z81133) Similarity to Human mRNA
product KIAA0077 (TR:Q14997)~cDNA
            EST yk243h8.5 comes from this gene~cDNA EST yk243h8.3
            comes from this gene~cDNA EST yk359h4.5 comes from this
            gene [Caenorhabditis elegans]
          Length = 1779

 Score = 32.3 bits (72), Expect = 4.1
 Identities = 44/182 (24%), Positives = 76/182 (41%), Gaps = 36/182
(19%)

Query: 2    DTLFNSTEKNARGIPQAPFIENVNEIIKDPSDFELCFNKFQERLSKYKFMQESK------
55
            D+L  + +K    IP+   +++   I    SD  LC    ++R  +    ++SK
Sbjct: 1462 DSLDTTIQKMLDKIPKLKLVKDQMMIRSSLSDSALCSPAEKKRCRRSSDSEDSKSMTYFR
1521

Query: 56   --LATIKQ-----LKTRIPDLENTLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQ
108
              L TI Q      K+  P L   +K+   L  +++E D          Y L++  Y
Sbjct: 1522 ALLETITQHYGSSTKSWSPIL---VKLLPKLMEYANEDD---------YDLSEETY--RD
1567

Query: 109  VDIPEDRA----DLKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEFLRE
164
            VDI ++ A    D     W+G +V     +DE +E LKK       S  V    ++F++
Sbjct: 1568 VDITQNSALIIHDYMSVSWIGNEV-----LDEIVETLKKTFTFHSGSWRVRLAVIKFVQA
1622

Query: 165  NI 166
            ++
Sbjct: 1623 SV 1624
>ref|NP_045678.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=11496896&dopt=GenPept> antigen, S1 [Borrelia burgdorferi]
```

```
 pir||E70207
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07463025&dopt=GenPept> antigen S1 - Lyme disease spirochete
plasmid A/lp54
 gb|AAC66229.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=02690228&dopt=GenPept> (AE000790) antigen, S1 [Borrelia
burgdorferi]
          Length = 417

 Score = 32.0 bits (71), Expect = 5.4
 Identities = 38/142 (26%), Positives = 66/142 (45%), Gaps = 17/142
(11%)

Query: 43  ERLSKYKFMQESKLATIKQL-KTRIPDLENTLK-ICQSLRNHSDEGDESDEPILLHYQLN
100
            E L KY++ ++      +  + K R  DL + +  + + + N+SD+ D  DE  LL   L
Sbjct: 174 EVLVKYRYSEKDVNQFLIDIGKKRWGDLSSKMSTLVRLIGNYSDKSDREDEISLLDMNLC
233

Query: 101 DTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKKLADSEQ-SLTVSTEDV
159
              Y   +++     AD+ V          LE  ID+ I  + K+L + +  SLT  +E
Sbjct: 234 QQFYL-TKINAGGSSADILVA--------LEKTIDQQISGVSKELLELKNFSLTTKSELD
284

Query: 160 EFL--RENITTME---VNCARL 176
             +L  + N+T  E   + C R+
Sbjct: 285 WYLNWKRNLTDEEEETLQCCRV 306
>pir||T41580
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07492541&dopt=GenPept> probable dna-binding protein -
fission yeast  (Schizosaccharomyces
           pombe)
 emb|CAB11611.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=02370467&dopt=GenPept> (Z98951) hypothetical protein
[Schizosaccharomyces pombe]
 emb|CAA20863.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=04239673&dopt=GenPept> (AL031546) putative dna-binding
protein [Schizosaccharomyces pombe]
          Length = 660

 Score = 32.0 bits (71), Expect = 5.4
 Identities = 35/156 (22%), Positives = 68/156 (43%), Gaps = 19/156
(12%)

Query: 19  PFIENVNEIIKDPSDFELCFNKFQERLSKY--KF----MQESKLATIKQLKTR-IPDLEN
71
            P+I     ++K   D   CF   Q R+ +   KF      +SKL   +++K R + DLEN
Sbjct: 427 PYISMFERLVKSQGDLVKCFLNIQYRMHELISKFPSDTFYDSKLVPAEEVKKRLLMDLEN
486

Query: 72  T---------LKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGL
122
                    +    +L N+ +  D+  E +   YQ + + + +AQ+        L+ GL
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Sbjct: 487 VEETELTDSPIYFYDTLGNYQE--DDRSEDMQNFYQDSKSNHWEAQIVSYHISGLLEAGL
544

Query: 123 WLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTED 158
               D+ +   P +   + L+++ L +     + + + D
Sbjct: 545 -EAKDIAVVTPYNAQVALIRQLLKEKGIEVEMGSVD 579
>pir||G81678
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=11277216&dopt=GenPept> amino acid ABC transporter,
periplasmic amino acid-binding protein
          TC0660 [imported] - Chlamydia muridarum (strain Nigg)
 gb|AAF39483.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07190695&dopt=GenPept> (AE002334) amino acid ABC
transporter, periplasmic amino
          acid-binding protein [Chlamydia muridarum]
         Length = 259

 Score = 32.0 bits (71), Expect = 5.4
 Identities = 36/135 (26%), Positives = 66/135 (48%), Gaps = 9/135 (6%)

Query: 21  IENVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLR
80
           I+++  + KD     L  +++     +    QE  L ++  +  RI   ++TL++   +
Sbjct: 123 IKSLVLVFKDGDSKSLPLDQYNSVAVQTGTYQEEYLQSLPGV--RIRSFDSTLEVLMEVL
180

Query: 81  NHSDEGDESDEPILLHYQLND--TLYTKAQVDIPEDRADLKVGLWLGAD-VMLEYPIDEA
137
            HS       EP +    L D  TL T+  +D+PED+  L  G+ + +D   L   I+ A
Sbjct: 181 -HSKSPIAVLEPSIAQVVLKDFPTLTTET-IDLPEDKWVLGYGIGVASDRPSLASDIEAA
238

Query: 138 IELLKKK--LADSEQ 150
           ++ +KK+  LA+ EQ
Sbjct: 239 VQEIKKEGVLAELEQ 253
>pir||B70438
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07517464&dopt=GenPept> hypothetical protein aq_1596 -
Aquifex aeolicus
 gb|AAC07503.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=02983955&dopt=GenPept> (AE000747) putative protein [Aquifex
aeolicus]
         Length = 227

 Score = 32.0 bits (71), Expect = 5.4
 Identities = 36/172 (20%), Positives = 71/172 (40%), Gaps = 24/172
(13%)

Query: 21  IENVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQS--
78
           +E   ++++    +E   + F+E   K  F  +++   IK+L+ R+ +L     I  S
Sbjct: 36  LEEREKLLETIRSYEEKLDSFEEEKQKMYFEIKNREEKIKELEQRLNELSEKGSISLSLS
95

Query: 79  ------LRNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEY
```

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132
                L N  +E  E  E  L  + L  + Y      + ED  ++
Sbjct: 96  KSIEEYLSNLKEEYREKVEEFLKTFLLEFSYYVPQVKVLKEDLRNI--------------
141

Query: 133 PIDEAIEL-LKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQR 183
            IDE I+     KL  + + L    ++++ L+EN+    +N   L + D+++
Sbjct: 142 -IDELIKFKTNLKLYINPEDLRYLNDELKSLKENLKAEGINLQVLEDKDLEK 192
>gb|AAG32647.1|AF209034_1
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=11181588&dopt=GenPept> (AF209034) 4b protein [Lettuce
necrotic yellows virus]
          Length = 306

 Score = 32.0 bits (71), Expect = 5.4
 Identities = 20/78 (25%), Positives = 36/78 (45%), Gaps = 6/78 (7%)

Query: 9   EKNARGIPQAPFIENVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPD
68
           E  A  I Q     N ++II  PSD ++ +       SK     + +    + ++TR+PD
Sbjct: 214 ELGAEDIDQLSLSYNDSKIISLPSDEDIYYR------SKGSLFSKGRTIKRRTMRTRVPD
267

Query: 69  LENTLKICQSLRNHSDEG 86
            E  +K+ +S  +  + G
Sbjct: 268 PEEPIKLTKSQSSRIEHG 285
>pir||T48658
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=11359188&dopt=GenPept> kinesin-like protein 1 - fission
yeast (Schizosaccharomyces pombe)
 gb|AAB88235.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01498677&dopt=GenPept> (U63916) kinesin-like protein 1
[Schizosaccharomyces pombe]
          Length = 832

 Score = 32.0 bits (71), Expect = 5.4
 Identities = 39/165 (23%), Positives = 75/165 (44%), Gaps = 18/165
(10%)

Query: 40  KFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQSLRNHSDEGDESDEPI-LLHYQ
98
           K QE   +   +      T+K+LK RI +LE  +K  +S +++S++  E +E I  L  +
Sbjct: 331 KQQEENDRISHIDYENDLTVKKLKRRISELEMAVKEYESEKSYSEK--EYEEKISSLRIE
388

Query: 99  LNDTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTED
158
           L D L   A++D+  ++         L  +    +   E +E L K +A  +     + ++
Sbjct: 389 LEDKL---AEIDMLRNK--------LLKEEHKHHSTSEKLEELSKYVASIQDKERNNGQN
437

Query: 159 VEFLRENITTMEVNCARLYN----WDVQRRQDLKQAQEGTKNLKI 199
              L+  I  +E     +YN     ++ RR+     QE   N+++
Sbjct: 438 ALELQARIQQLERRNEDMYNKLLAEEIIRRKLHNDIQELKGNIRV 482
>pir||I40296
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
```

n&list_uids=02120496&dopt=GenPept> S1 protein - Lyme disease spirochete
gb|AAA81351.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=01063417&dopt=GenPept> (L34016) S1 gene product [Borrelia
burgdorferi]
Length = 417

Score = 32.0 bits (71), Expect = 5.4
Identities = 38/142 (26%), Positives = 66/142 (45%), Gaps = 17/142
(11%)

```
Query: 43  ERLSKYKFMQESKLATIKQL-KTRIPDLENTLK-ICQSLRNHSDEGDESDEPILLHYQLN
100
           E L KY++ ++      +  + K R  DL + +  + + + N+SD+ D  DE  LL   L
Sbjct: 174 EVLVKYRYSEKDVNQFLIDIGKKRWGDLSSKMSTLVRLIGNYSDKSDREDEISLLDMNLC
233

Query: 101 DTLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKKLADSEQ-SLTVSTEDV
159
              Y   +++     AD+ V         LE  ID+ I  + K+L + +  SLT  +E
Sbjct: 234 QQFYL-TKINAGGSSADILVA--------LEKTIDQQISGVSKELLELKNFSLTTKSELD
284

Query: 160 EFL--RENITTME---VNCARL 176
            +L  + N+T  E   + C R+
Sbjct: 285 WYLNWKRNLTDEEEETLQCCRV 306
>pir||H72208
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07462330&dopt=GenPept> conserved hypothetical protein -
Thermotoga maritima (strain MSB8)
gb|AAD36874.1|AE001818_9
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=04982392&dopt=GenPept> (AE001818) conserved hypothetical
protein [Thermotoga maritima]
Length = 717

Score = 32.0 bits (71), Expect = 5.4
Identities = 22/71 (30%), Positives = 34/71 (46%), Gaps = 7/71 (9%)

Query: 13  RGIPQAPFIENVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENT
72
           RG+     IE+V   +KD  D    F K  E+L++ K+    ++ T K L+   PD
Sbjct: 316 RGLSLHLVIESVEFSVKDVEDMSKVFKKIGEKLNERKY----RMYTEKDLEAIFPD---D
368

Query: 73  LKICQSLRNHS 83
           L + Q   NH+
Sbjct: 369 LNLIQEKGNHT 379
>pir||T22976
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07504717&dopt=GenPept> hypothetical protein F59A2.6 -
Caenorhabditis elegans
emb|CAA84332.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=03877858&dopt=GenPept> (Z34801) Similarity with drosohila
MSP-300 protein (PIR acc. no.
           S30431), contains similarity to Pfam domain: PF01465
```

(GRIP domain), Score=90.2, E-value=1.4e-23, N=1
[Caenorhabditis elegans]
emb|CAA91344.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=03878093&dopt=GenPept> (Z66514) Similarity with drosohila MSP-300 protein (PIR acc. no.
S30431), contains similarity to Pfam domain: PF01465
(GRIP domain), Score=90.2, E-value=1.4e-23, N=1
[Caenorhabditis elegans]
Length = 1133

Score = 31.6 bits (70), Expect = 7.0
Identities = 21/64 (32%), Positives = 38/64 (58%), Gaps = 3/64 (4%)

Query: 135 DEAIELLKKKLADSEQSLTVSTEDVEFLRENITTMEVNCARLYNWDVQRRQDLKQAQEGT 194
DEA+ELLK+KL + E++++ + L E+ T+ A ++ ++Q L++AQ
Sbjct: 210 DEAVELLKQKLEEVEKNMSDVEVQKQLLLESTTSEMKQHAEAA--EIVKKQ-LEEAQSSI 266

Query: 195 KNLK 198
+NLK
Sbjct: 267 ENLK 270
>pir||T41342
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=07492491&dopt=GenPept> probable coiled-coil protein - fission yeast (Schizosaccharomyces
pombe)
emb|CAA22653.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=04107284&dopt=GenPept> (AL035076) putative coiled-coil protein [Schizosaccharomyces pombe]
Length = 1115

Score = 31.6 bits (70), Expect = 7.0
Identities = 35/128 (27%), Positives = 59/128 (45%), Gaps = 12/128 (9%)

Query: 45 LSKYKFMQESKLAT--IKQLKTRIPDLENTL-KICQSLRNHSDEGDESDEPILLHYQLND 101
+S+ K ++S T KQL+ D EN L ++ Q R D+ E E I +L D
Sbjct: 493 VSRMKTQEQSIELTRLYKQLQDIEEDYENKLMRMEQQWREDVDQLQEYVEEIT--QELQD 550

Query: 102 TLYTKAQVDIPEDRADLKVGLWLGADVMLEYPIDEAIELLKKKLADSEQSLTVSTEDVEF 161
T ++ D + VG L + IE +K + ++E+S++ E+VE
Sbjct: 551 TKEVLSKSSKESDDYEEVVG-------KLRTEAEREIEKFEKTIRENEESISLFKEEVEK 603

Query: 162 LRENITTM 169
L + IT +
Sbjct: 604 LTDEITQL 611
>gb|AAD33718.1|AF136711_1
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=04928755&dopt=GenPept> (AF136711) myosin heavy chain [Amoeba proteus]
Length = 2138

```
 Score = 31.2 bits (69), Expect = 9.2
 Identities = 23/101 (22%), Positives = 47/101 (45%), Gaps = 7/101 (6%)

Query: 21   IENVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQLKTRIPDLENTLKICQ-SL
79
            + NV ++  +  D    +    + LSK     +   A +K+L+  + + +++L   +  L
Sbjct: 1415 VRNVKKVQDEVEDLNEQYENASKELSKLDKGNKKTEAELKELRRHVQESQSSLDAGELKL
1474

Query: 80   RNHSDEGDESDEPILLHYQLNDTLYTKAQVDIPEDRADLKV 120
            R+  DE DE       LH+QL D      + ++   + +  L+V
Sbjct: 1475 RHTQDELDE------LHHQLEDLEAKSSSLERSKKQLQLQV 1509
>emb|CAA03928.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=02598551&dopt=GenPept> (AJ000109) carbamoylphosphate
synthetase [Lactococcus lactis]
          Length = 1064

 Score = 31.2 bits (69), Expect = 9.2
 Identities = 30/101 (29%), Positives = 49/101 (47%), Gaps = 4/101 (3%)

Query: 64  TRIP--DLENTLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTK-AQVDIPEDRADLKV
120
            T IP   L   + + ++L++   E   +  P ++H +     +TK A+VD
Sbjct: 848 TNIPMAQLATQMILGENLKDLGYEAGLAPTPDMVHVKAPVFSFTKLAKVDSLLGPEMKST
907

Query: 121 GLWLGADVMLEYPIDEAIELLKKKLADSEQSL-TVSTEDVE 160
            GL +G+DV LE  + ++ E  K  +AD    L TV+ ED E
Sbjct: 908 GLAMGSDVTLEKALYKSFEAAKLHMADYGSVLFTVADEDKE 948
>pir||F72363
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07462655&dopt=GenPept> hypothetical protein - Thermotoga
maritima (strain MSB8)
 gb|AAD35622.1|AE001729_2
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=04981051&dopt=GenPept> (AE001729) hypothetical protein
[Thermotoga maritima]
          Length = 758

 Score = 31.2 bits (69), Expect = 9.2
 Identities = 42/207 (20%), Positives = 90/207 (43%), Gaps = 31/207
(14%)

Query: 1   MDTLFNSTEKNA-------RGIPQAPFIENV-NE----IIKDPSDFELCFNKFQERLSKY
48
            +D   N ++K A       R  P+A  +E + NE     ++K   + E   +  +ER+  +
Sbjct: 98  VDNTLNGSKKVASFLMESYRNRPEAVELERILNEDFSVLMKKTKELEAEISNLKERVEAW
157

Query: 49  KFMQESKLATIKQLKTRIPDLENTLKICQSLRNHSDEGDESDEPILLHYQLNDTLYTKAQ
108
            K  + S L  +K+ K    DL+    ++ +   +   E ++S+    +  ++N+      +
Sbjct: 158 KEKRRSLLLVLKRKKELSRDLQEKRRLLEEEIDRF-ESEKSERLSSIEARINEVKAELLR
216
```

```
Query: 109 VD-----------IPEDRADLKVGLWLGADVMLE--YPIDEAIELLKKKLADSEQSLTVS
155
           V+           +PE++    + L    D + E    +   IE L++K  D+E+ L
Sbjct: 217 VEKELEEIERETAVPEEKVREAIELAQKLDYLRERGEELKREIESLEEKSKDTEERLKTI
276

Query: 156 TEDVEFLRENITTMEVNCARLYNWDVQ 182
            +D      +++++E    +L N  +Q
Sbjct: 277 MKDF-----SVSSLEELKLKLENMKLQ 298
>pir||T49451
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=11359561&dopt=GenPept> kinesin-like protein Kif21a related
protein [imported] - Neurospora
            crassa
 emb|CAB91712.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07899307&dopt=GenPept> (AL356173) kinesin-like protein
Kif21a related protein [Neurospora
            crassa]
          Length = 1742

 Score = 31.2 bits (69), Expect = 9.2
 Identities = 18/63 (28%), Positives = 33/63 (51%), Gaps = 4/63 (6%)

Query: 132  YPIDEAIELLKKKLADSEQSLTVSTEDVEFLRE----NITTMEVNCARLYNWDVQRRQDL
187
            YP+ E++ L+KKK+ D E      ++  VE L +    N   +++   RL     ++ Q L
Sbjct: 1459 YPVKESVALVKKKMVDLETKNKKNSRLVEELEDQLQSNYDQVQITSNRLSMLQSEKTQQL
1518

Query: 188  KQA 190
            ++A
Sbjct: 1519 EEA 1521
>pir||F71962
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=07464634&dopt=GenPept> hypothetical protein jhp0191 -
Helicobacter pylori (strain J99)
 gb|AAD05774.1|
<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=Protei
n&list_uids=04154710&dopt=GenPept> (AE001457) putative [Helicobacter
pylori J99]
          Length = 916

 Score = 31.2 bits (69), Expect = 9.2
 Identities = 40/184 (21%), Positives = 78/184 (41%), Gaps = 16/184 (8%)

Query: 3   TLFNSTEKNARGIPQAPFIENVNEIIKDPSDFELCFNKFQERLSKYKFMQESKLATIKQL
62
           +LFN T  N   I   P   N    IK   D E+ F+  +      K++QE    ++K++
Sbjct: 440 SLFNKTNFNPNKI-WIPLEFNKRSKIKFDKDLEIYFDSHESFNISKKYLQEIDQESLKKI
498

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associated protein - fission yeast
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 emb|CAA19588.1|
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S1: 42 (22.0 bits)
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Bottom of Form 1

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A novel protein complex promoting formation of functional α- and γ-tubulin

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We describe the identification of *GIM1/YKE2*, *GIM2/PAC10*, *GIM3*, *GIM4* and *GIM5* in a screen for mutants that are synthetically lethal with *tub4-1*, encoding a mutated yeast γ-tubulin. The cytoplasmic Gim proteins encoded by these *GIM* genes are present in common complexes as judged by co-immunoprecipitation and gel filtration experiments. The disruption of any of these genes results in similar phenotypes: the *gim* null mutants are synthetically lethal with *tub4-1* and super-sensitive towards the microtubule-depolymerizing drug benomyl. All except Δ*gim4* are cold-sensitive and their microtubules disassemble at 14°C. The Gim proteins have one function related to α-tubulin and another to Tub4p, supported by the finding that the benomyl super-sensitivity is caused by a reduced level of α-tubulin while the synthetic lethality with *tub4-1* is not. In addition, *GIM1/YKE2* genetically interacts with two distinct classes of genes, one of which is involved in tubulin folding and the other in microtubule nucleation. We show that the Gim proteins are important for Tub4p function and bind to overproduced Tub4p. The mammalian homologues of *GIM1/YKE2* and *GIM2/PAC10* rescue the synthetically lethal phenotype with *tub4-1* as well as the cold-sensitivity and benomyl super-sensitivity of the yeast deletion mutants. We suggest that the Gim proteins form a protein complex that promotes formation of functional α- and γ-tubulin.
Keywords: chaperonin/spindle pole body/TRiC/Tub4p/γ-tubulin

Introduction

Microtubules are hollow cylinders with a diameter of 25 nm. They are part of the cytoskeleton of eukaryotic cells and play an important role in numerous cellular processes, including chromosome segregation in mitosis and meiosis, organelle positioning and intracellular transport. The wall of the microtubule cylinder consists of 13 protofilaments which are strings of alternating α- and β-tubulin subunits pointing in the same direction (reviewed by Mandelkow and Mandelkow, 1993). Therefore, microtubules are polar structures with biochemically distinct ends. Microtubule formation is a complex process that includes the post-translational folding of α- and β-tubulin, their assembly into the heterodimer tubulin and finally the formation of microtubules from tubulin subunits (Solomon, 1991). All of these steps are assisted by proteins *in vivo*.

The first post-translational step in the pathway leading to the formation of the tubulin heterodimer is the folding of α- and β-tubulin. Genetic (Ursic and Culbertson, 1991; Chen *et al.*, 1994; Vinh and Drubin, 1994; Archer *et al.*, 1995) and biochemical (Frydman *et al.*, 1992; Yaffe *et al.*, 1992; Melki *et al.*, 1993; Sternlicht *et al.*, 1993; Tian *et al.*, 1996) evidence suggests that tubulins undergo facilitated folding via their interaction with cytoplasmic chaperonins. Conditional lethal mutations in *TCP1*, *BIN2*, *BIN3* and *ANC2*, coding for subunits of cytoplasmic chaperonin (TRiC), affect microtubule as well as actin functions (Ursic and Culbertson, 1991; Chen *et al.*, 1994; Li *et al.*, 1994; Miklos *et al.*, 1994; Vinh and Drubin, 1994). For example, *tcp1-1* cells are cold-sensitive for growth, and accumulate multi-nucleated and anucleated cells with abnormal microtubule structures. This mutant is also sensitive towards the microtubule-depolymerizing drug benomyl (Ursic and Culbertson, 1991). Remarkably, *bin3-1* cells display a normal distribution of unbudded, small-budded and large-budded cells, although the microtubules of *bin3-1* cells disassemble at 14°C (Chen *et al.*, 1994).

While the cytoplasmic chaperonin is sufficient for the production of native actin in an ATP-dependent manner (Gao *et al.*, 1992), folding of α- and β-tubulin requires the participation of additional cofactors. Such proteins, named cofactors A, B, C, D and E, have been identified by biochemical approaches (Tian *et al.*, 1996, 1997). Potential yeast homologues of cofactors A, B, D and E are Rbl2p, Alf1p, Cin1p and Pac2p (Archer *et al.*, 1995; Tian *et al.*, 1996; Geiser *et al.*, 1997). Surprisingly, *ALF1*, *RBL2*, *CIN1* and *PAC2* are not essential for growth of yeast cells (Hoyt *et al.*, 1990; Stearns *et al.*, 1990; Archer *et al.*, 1995). However, their deletion causes sensitivity towards benomyl and they show multiple genetic interactions with genes coding for components of the microtubule system. In addition, Rbl2p interacts directly with yeast β-tubulin (Archer *et al.*, 1995).

In many cell types, microtubule assembly is initiated at centrosomes, basal bodies, spindle pole bodies (SPBs) or nucleus-associated bodies, structures for which Pickett-Heaps (1969) coined the generic term microtubule-organizing centre (MTOC). MTOCs assist the assembly of the first tubulin monomers into oligomers, a step known as microtubule nucleation (Mitchison and Kirschner, 1984). A universal component of MTOCs involved in microtubule nucleation is γ-tubulin (Oakley *et al.*, 1990; Horio *et al.*, 1991; Stearns *et al.*, 1991). γ-Tubulin forms a complex with additional proteins (Stearns and Kirschner, 1994;

Moudjou *et al.*, 1996; Akashi *et al.*, 1997), and purification of such a complex from *Xenopus laevis* eggs identified α-, β- and γ-tubulin and proteins with mol. wts of 195, 133, 109 and 75 kDa (Zheng *et al.*, 1995).

In the yeast *Saccharomyces cerevisiae*, the MTOC is known as the SPB. It is a multi-layered structure which is embedded in the nuclear envelope (Byers and Goetsch, 1975; Byers, 1981). The yeast γ-tubulin is encoded by the essential *TUB4* gene (Sobel and Snyder, 1995; Marschall *et al.*, 1996; Spang *et al.*, 1996). Tub4p forms a 6S complex with the SPB components Spc98p and Spc97p (Geissler *et al.*, 1996; Spang *et al.*, 1996; Knop *et al.*, 1997). Purification of this complex suggests that it contains only one molecule of Spc98p and Spc97p, but two or more molecules of Tub4p (Knop and Schiebel, 1997). Fractionation experiments suggest that the Tub4p complex assembles in the cytoplasm followed by its nuclear import via an essential nuclear localization sequence in Spc98p (G. Pereira *et al.*, 1998). Finally, Spc98p and Spc97p of the Tub4p complex interact in the nucleus with the amino-terminal domain of the SPB component Spc110p (Knop and Schiebel, 1997). The binding site for the Tub4p complex at the outer plaque is still unknown.

To identify genes that are involved in yeast γ-tubulin functions, we performed a genetic screen for mutants that are synthetically lethal with *tub4-1*. We identified *SPC98* and *SPC97* coding for components of the yeast γ-tubulin complex and five additional genes, which we named *GIM1–GIM5*. Their products are not associated with the SPB, instead they form cytoplasmic multi-protein complexes which promote formation of functional α-tubulin and Tub4p. Most interestingly, the Gim proteins are phylogenetically conserved proteins, and mouse and human homologues function in yeast, indicating that the mammalian proteins fulfil a very similar role.

Results

Identification of GIM1/YKE2, GIM2/PAC10, GIM3, GIM4 and GIM5 in a screen for mutants that are synthetically lethal with a mutated yeast γ-tubulin

In the yeast *S.cerevisiae*, the γ-tubulin Tub4p forms a complex with the SPB components Spc98p and Spc97p. Physical interaction between Tub4p, Spc98p and Spc97p is reflected by multiple genetic interactions, including synthetic lethality (Geissler *et al.*, 1996; Knop *et al.*, 1997). The latter is an indication of a functional relationship of two gene products. To identify further components that functionally interact with *TUB4*, we performed a genetic screen for mutants that are synthetically lethal with *tub4-1*, yielding 12 mutants with this phenotype. Subsequent analysis showed that two of these mutants were defective in *SPC98* and two in *SPC97*. The other eight belong to five complementation groups which we named *GIM1–GIM5* (genes involved in microtubule biogenesis). Plasmids containing *GIM1–GIM5* were isolated by transforming the mutants with a yeast genomic library. Subcloning of DNA fragments and complementation analysis showed that *GIM1* is identical to *YKE2* (Shang *et al.*, 1994), coding for a protein of 114 amino acids (Table I). *GIM2* had already been identified as *PAC10*, a gene that becomes essential in the absence of the *CIN8*-encoded kinesin motor (Geiser *et al.*, 1997). *GIM3* corresponds to the open reading frame (ORF) YNL153c, encoding a protein of 129 amino acids. The DNA fragment complementing *gim4* cells contained three overlapping ORFs, one of which was interrupted by an intron. The various coding regions were cloned directly behind the *ADH* promoter of plasmid p415-*ADH*. Only the *ADH* promoter fusion with the intron-containing ORF YEL003w complemented *gim4* cells (data not shown). Gim4p is a protein of 132 amino acids. *GIM5* (ORF YML094w) also contains a small intron and encodes a protein of 163 amino acids.

The Gim proteins are phylogenetically conserved coiled-coil proteins

As reported for Gim2p/Pac10p (Geiser *et al.*, 1997), all Gim proteins have a high probability of forming coiled-coils (Lupas, 1996). Sequence comparisons have shown that Gim1p/Yke2p, Gim2p/Pac10p, Gim3p, Gim4p and Gim5p are related proteins. They also have relatives in mammals, *Caenorhabditis elegans* and *Schizosaccharomyces pombe*, revealing that they are phylogenetically conserved (Figure 1). Remarkably, Gim1p/Yke2p and Gim5p are homologous to two proteins of the archaebacterium *Methanococcus jannaschii*, while no relatives were found in eubacteria. We also noticed that the Gim proteins are more closely related to their homologues from other species than to each other (Figure 1).

Deletion of the GIM genes causes microtubule defects

To understand the function of the *GIM* gene products, we investigated whether they are essential for growth of yeast cells. We disrupted the entire coding regions of the *GIM* genes in the diploid yeast strain YPH501. As shown for Δ*gim5* (Figure 2A), spore analysis revealed that *GIM1/YKE2*, *GIM3*, *GIM4* and *GIM5* are not essential for growth. In agreement with a previous report (Geiser *et al.*, 1997), we observed that Δ*pac10* cells were defective in spore germination. Such a defect was not apparent for the other *GIM* mutants, raising the possibility that *GIM2/PAC10* has an additional specialized function in spore germination. The disruption of any of the *GIM* genes resulted in a slow growth phenotype at 30°C (Figure 2A and B). A more detailed analysis indicated that the doubling time of Δ*gim1/yke2*, Δ*gim2/pac10*, Δ*gim3* and Δ*gim5* cells grown in liquid medium at 30°C was increased by a factor of 1.4 compared with the wild-type, while the doubling time of Δ*gim4* cells was only affected 1.1-fold. It has been reported previously that a deletion mutant of *GIM1/YKE2* shows normal growth over a wide range of temperatures (Shang *et al.*, 1994). This discrepancy with our study is explained either by strain differences or by the fact that Shang *et al.* (1994) did not disrupt the entire coding region of *YKE2*.

Common phenotypes of mutants affecting the microtubule cytoskeleton are a cold-sensitive growth defect and an increased sensitivity towards the microtubule-depolymerizing drug benomyl (Neff *et al.*, 1983; Huffaker *et al.*, 1988). Therefore, we investigated whether the growth defects of the haploid *gim* null strains were intensified at lower temperatures. The *gim* null strains showed an enhanced growth defect at reduced temperatures in comparison with the wild-type (Figure 2B; compare 30°C with 23°C and 14°C plates). An exception was

Table I. Properties of the *GIM* genes and the corresponding deletion mutants

	ORF name	Amino acids in encoded protein	Cold-sensitive[a]	Benomyl-sensitive[b] (2.5 μg/ml)	Relative volume[c]	Suppression of benomyl super-sensitivity[d]
GIM1/YKE2	YLR200w	114	yes	yes	2.4	yes
GIM2/PAC10	YGR078c	199	yes	yes	2.2	yes
GIM3	YNL153c	129	yes	yes	2.4	yes
GIM4	YEL003w	132	no	yes	2.4	yes
GIM5	YML094w	163	yes	yes	2.3	yes

[a]Cold sensitivity was determined as described in the legend to Figure 2B.
[b]Benomyl sensitivity was determined as in Figure 2C.
[c]Determination of cell volumes (compared with wild-type) is described in Materials and methods.
[d]Suppression of benomyl super-sensitivity by overexpression of *TUB1* or *RBL2* was determined as in Figure 5C.

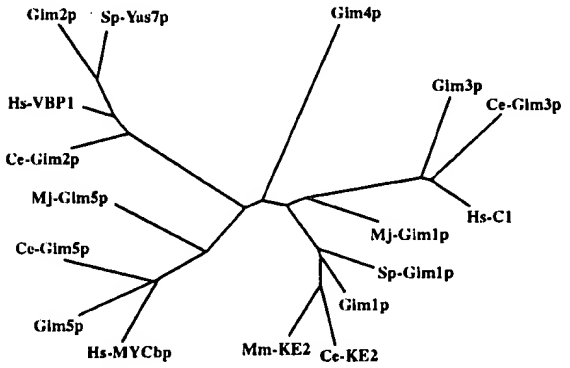


Fig. 1. *GIM1/YKE2*, *GIM2/PAC10*, *GIM3*, *GIM4* and *GIM5* encode phylogenetically conserved proteins. Dendrogram of the Gim proteins and their homologous proteins from *S.pombe* (Sp), *C.elegans* (Ce), mouse (Mm), human (Hs) and *M.jannaschii* (Mj) generated by the PROTDIST and FITCH modules of PHYLIP (Felsenstein, 1996). The dendrogram is based on an alignment by A.Lupas (personal communication) using MACAW (Schuler *et al.*, 1991). Accession Nos: Sp-Gim1p, Z99260; Mm-KE2, I53651; Ce-KE2, P52554; Mj-Gim1p, C64423; Sp-Yas7p, Q10143; Ce-Gim2p, Z81587; Hs-VBP-1, U56833; Ce-Gim3p, Z73102; Hs-C1, U41816; Ce-Gim5p, U00036; Hs-MYCbp, Q99471; Mj-Gim5p, H64418.

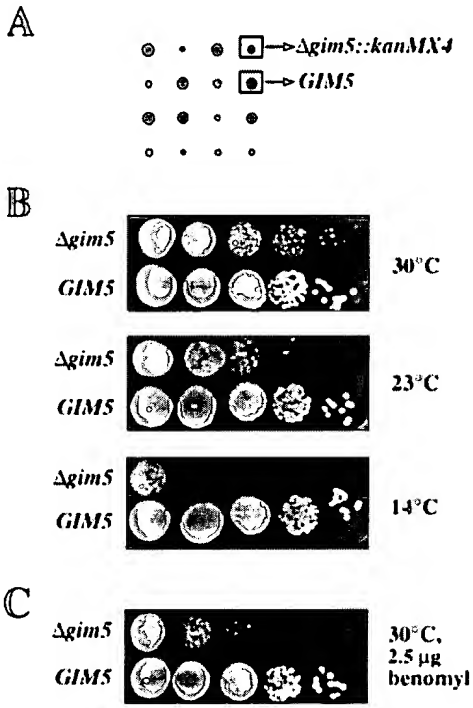


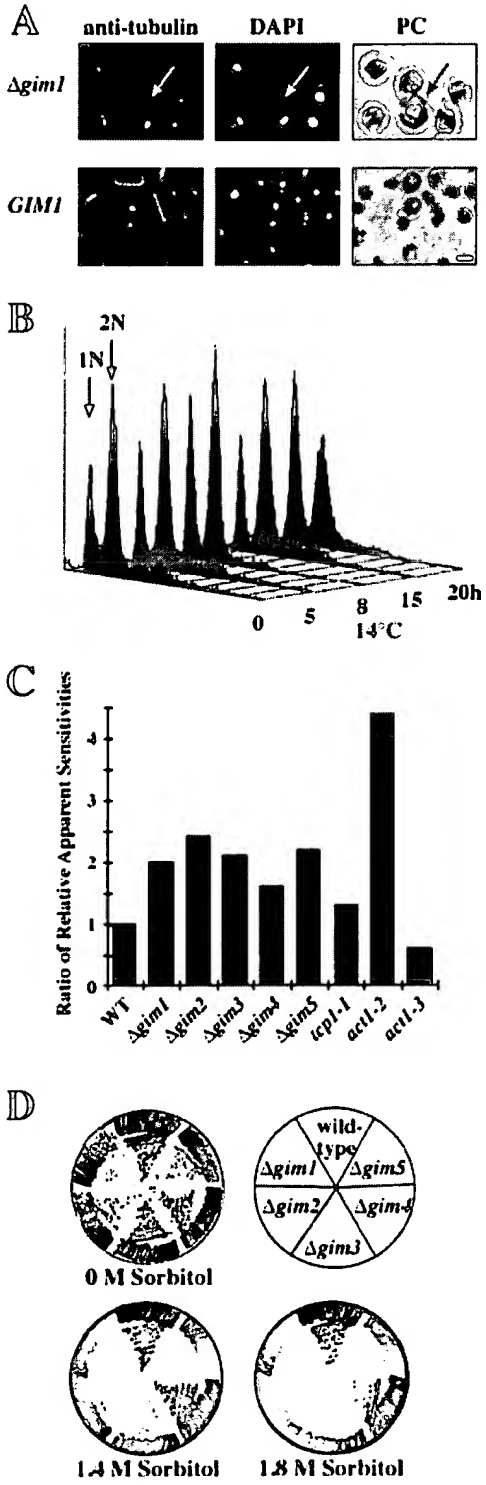
Fig. 2. Δ*gim5* cells are cold- and benomyl-sensitive. (**A**) Tetrads of a diploid *GIM5/Δgim5::kanMX4* strain were analysed for growth at 30°C on YPD plates. All spores from a tetrad germinated and formed colonies. These colonies were tested for growth on YPD plates containing the kanamycin derivative G418. Only cells which carry the *kanMX4* gene grow on these plates. Two of the spores of each tetrad, which were G418 resistant (Δ*gim5::kanMX4*), grew more slowly in comparison with the G418-sensitive *GIM5* cells. (**B**) The growth defect of Δ*gim5::kanMX4* cells on YPD plates is more pronounced at lower temperatures. Serial dilutions of Δ*gim5* and *GIM5* cells were grown on YPD plates. One plate was incubated for 3 days at 30°C. The plates at 23 and 14°C were incubated until the sizes of the *GIM5* colonies were approximately the same as on the 30°C plate. (**C**) Δ*gim5::kanMX4* cells are super-sensitive towards the microtubule-depolymerizing drug benomyl. Serial dilutions of Δ*gim5::kanMX4* and *GIM5* cells were grown on YPD plates containing 2.5 μg/ml benomyl at 30°C.

Δ*gim4* cells, which did not show an enhanced growth defect at lower temperatures (Table I). In contrast to the wild-type, all *gim* null strains were super-sensitive to 2.5 μg/ml benomyl (Figure 2C). Such a strong benomyl sensitivity has only been observed for mutants defective in microtubule biogenesis (Stearns *et al.*, 1990; Chen *et al.*, 1994; Archer *et al.*, 1995; Tian *et al.*, 1997).

We investigated whether the Gim proteins function in parallel pathways. In this case double mutants should have more pronounced defects compared with the single mutants. However, the double deletion mutants of Δ*gim1/yke2* together with any one of Δ*gim2/pac10*, Δ*gim3*, Δ*gim4* or Δ*gim5* were viable and as benomyl-sensitive as the single mutants. We also tested whether overexpression of any of the *GIM* genes rescued the synthetically lethal phenotype of *gim tub4-1* cells. This was the case for *GIM1* which weakly suppressed the defect of *gim4 tub4-1* cells (data not shown).

The strong benomyl super-sensitivity of the haploid *gim* null strains suggests that the gene products are needed for either tubulin formation or microtubule stability. To analyse these possibilities, we investigated, by indirect immunofluorescence, the microtubule arrays of Δ*gim1/yke2*, Δ*gim2/pac10* and Δ*gim3* cells incubated at 14°C for 20 h. The phenotype is shown for Δ*gim1/yke2* cells as a representative, since it is very similar to that of the other mutants. Δ*gim* cells lost most of their cytoplasmic and nuclear microtubules, leaving only a small spot near the nucleus, most likely at the SPB (Figure 3A). This observation suggests that microtubule attachment to the SPB via the Tub4p complex is still taking place in Δ*gim* cells. Instead microtubule stability seems to be impaired. Furthermore, cells with a large bud (Figure 3A, arrow) had

only one 4',6'-diamidino-2-phenylindole (DAPI) staining region in one of the two cell bodies, a phenotype that is consistent with the observed defect in microtubule organization. Despite these defects, *gim* null cells incubated for 20 h at 14°C were still as viable as wild-type cells when then grown at 30°C (data not shown). Based on the distributions of the DNA content (Figure 3B) and of the cell morphologies (Figure 3A), Δ*gim* cells did not arrest at a defined stage of the cell cycle. This observation was surprising, since many mutants with a defect in spindle formation arrest in mitosis due to a mitotic check point (Hoyt *et al.*, 1990; Li and Murray, 1991). The non-arrest phenotype could point to a role for the Gim proteins in mitotic check point control. However, all *GIM* deletion mutants arrested in the cell cycle like wild-type cells in response to the microtubule-depolymerizing drug nocodazole, while the mitotic check point control mutant *bub2* did not (Hoyt *et al.*, 1990; Li and Murray, 1991; Geiser *et al.*, 1997; data not shown). This led us to conclude that the *GIM* gene products are not part of a mitotic check point. Taken together, the strong benomyl sensitivity and the microtubule defects of the *gim* null mutants at reduced temperature are most consistent with a role for the Gim proteins in tubulin biogenesis.



gim null mutants are sensitive towards the actin inhibitor latrunculin-A and are osmotically sensitive

We noticed that the volume of cells of the *GIM* deletion mutants incubated at 14°C was ~2.2- to 2.4-fold increased compared with the wild-type (Table I). Such a phenotype has been reported, among others, for mutants affecting the actin cytoskeleton (reviewed by Drubin, 1990). Many mutants with actin defects are sensitive towards the actin-binding drug latrunculin-A (Ayscough *et al.*, 1997) and are osmotically sensitive (Drubin, 1990). We found that the *gim* null strains were more sensitive towards latrunculin-A than the wild-type, which indicates an influence of the Gim proteins on the actin cytoskeleton (Figure 3C). This sensitivity was stronger compared with that of the cold-sensitive mutant *tcp1-1*, but was, however, clearly weaker compared with the actin mutant *act1-2*. *TCP1* encodes a component of TRiC which is required for actin folding (Ursic and Culbertson, 1991). Furthermore, similarly to actin mutants, Δ*gim1/yke2*, Δ*gim2/pac10* and Δ*gim5* cells did not grow on plates containing 1.4 M sorbitol. In contrast, Δ*gim3* cells grew slowly, while Δ*gim4* grew as wild-type cells (Figure 3D). At 1.8 M sorbitol, Δ*gim3* cells failed to grow and a weak growth defect of Δ*gim4* cells was observed (Figure 3D). We sought additional evidence for a role for the Gim proteins in actin function. However, deletion of *GIM1/YKE2* was synthetically lethal with neither *act1-2* nor *act1-3*. In addition, Δ*gim1/yke2*, Δ*gim2/pac10*, Δ*gim3* and Δ*gim4* cells did not show an obvious defect in actin organization as judged by indirect

Fig. 3. Microtubule defects of Δ*gim1/yke2* cells. **(A)** Microtubule staining of the *gim1/yke2* null mutant. Cells of Δ*gim1/yke2::kanMX4* and *GIM1/YKE2* were grown in YPD medium at 30°C. The cells were diluted to 5×10^6 cells/ml using pre-cooled YPD medium. The cultures were then incubated at 14°C for two doubling times (20 h). Microtubules were detected by indirect immunofluorescence with anti-tubulin antibodies. DNA was stained with DAPI. Cells were also inspected by phase contrast microscopy (PC). The arrows point to a Δ*gim1/yke2* cell with a large bud that contains no mitotic spindle and only one DAPI-staining region. Bar: 5 μm. **(B)** DNA content of Δ*gim1/yke2* cells. Δ*gim1/yke2* cells were incubated in YPD at 14°C. Samples were taken at the indicated time points. The DNA content of these cells was analysed by flow cytometry. **(C)** Sensitivity of the indicated strains towards latrunculin-A. Relative apparent sensitivity was determined as described (Ayscough *et al.*, 1997). **(D)** Sensitivity of *gim* null strains towards high osmolarity. Wild-type cells, Δ*gim1/yke2*, Δ*gim2/pac10*, Δ*gim3*, Δ*gim4* and Δ*gim5* cells were grown for 3 days at 30°C on YPD plates containing 0, 1.4 or 1.8 M sorbitol.

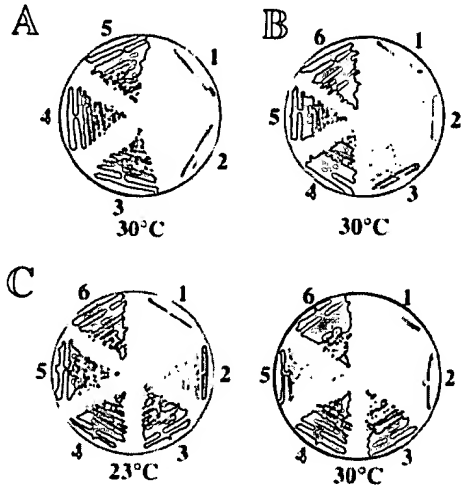


Fig. 4. Genetic interactions of *GIM1/YKE2* with *TUB4*, *SPC98* and *SPC97*. (**A**) *GIM1/YKE2* of strain ESM183 (Δ*tub4::HIS3* pRS316-*TUB4*) was disrupted using the *kanMX4* gene. The resulting strain SGY119 was unable to grow on 5-FOA plates which selects against the *URA3*-based plasmid (sector 1), confirming that *TUB4* is an essential gene (Spang *et al.*, 1996). However, strain SGY119 was able to grow on 5-FOA when it contained a *LEU2*-based plasmid carrying *TUB4* (sector 3), but not when it contained *tub4-1* (sector 2), indicating that Δ*gim1/yke2* is synthetically lethal with *tub4-1*. Controls established that *tub4-1* (sector 4) and Δ*gim1/yke2* cells (sector 5) grow on 5-FOA at 30°C. (**B**) In a similar manner as in (A), using strain ESM243 (Δ*spc98::HIS3* pRS316-*SPC98*), we tested for genetic interactions of *GIM1/YKE2* with *SPC98*. Strain SGY120 (ESM243 Δ*gim1/yke2*) was unable to grow on 5-FOA (sector 1), as *SPC98* is essential for growth (Geissler *et al.*, 1996). As expected, growth was observed in the presence of a *LEU2*-based plasmid containing *SPC98* (sector 4). In contrast, *spc98-2* (sector 2) and *spc98-1* (sector 3) did not or hardly support growth, indicating synthetic lethality and synthetic toxicity of Δ*gim1/yke2 spc98-2* (sector 2) and Δ*gim1/yke2 spc98-1* (sector 3). We established that *spc98-2* (sector 5) and *spc98-1* (sector 6) cells grow well on 5-FOA at 30°C. (**C**) *GIM1/YKE2* of strain YMK10 (Δ*spc97::HIS3* pRS316-*SPC97*) was disrupted. The resulting strain SGY121 was unable to grow on 5-FOA (sector 1), unless the plasmid pRS315-*SPC97* was present (sector 4). Since *spc97-20* Δ*gim1/yke2* cells barely grow at 23°C (sector 2), while cells of *spc97-20* (sector 5) grow well, we conclude that Δ*gim1/yke2* is synthetically toxic with *spc97-20*. In contrast, *spc97-14* combined with Δ*gim1/yke2* (sector 3) grow as *spc97-14* cells at 23 or 30°C (sector 6).

immunofluorescence using anti-actin antibodies (data not shown), suggesting that the *gim* null mutants have only subtle actin defects. In conclusion, the *GIM* genes may have multiple functions, since their deletion causes microtubule defects as well as an increased sensitivity towards the actin-specific drug latrunculin-A and towards high osmolarity.

The GIM genes interact genetically with genes involved in microtubule biogenesis

To gain further insight into the role of the *GIM* genes, we investigated their genetic interactions with other genes involved in microtubule function. We first addressed the question of whether total loss of *GIM* gene function is synthetically lethal with mutants of the Tub4p complex. Using the approach described in Figure 4A, we could show that this is indeed the case for *tub4-1*. This result was confirmed by tetrad analysis using a *GIM4/Δgim4::HIS3MX6 TUB4/tub4-1* strain. No viable temperature-sensitive *HIS3* spores were obtained, confirming that Δ*gim4::HIS3MX6* combined with *tub4-1* is lethal. Microscopic inspection of the non-colony-forming spores identified several hundred cells. Therefore, Δ*gim4 tub4-1* cells died only after multiple duplications. A similar result was obtained with *GIM3* (data not shown).

In a similar manner, we tested whether *GIM1/YKE2*, *GIM2/PAC10* and *GIM4* showed interactions with *SPC98* and *SPC97*, coding for components of the yeast γ-tubulin complex. Δ*gim1/yke2* was synthetically lethal with *spc98-2* and synthetically toxic when combined with *spc98-1* (Figure 4B). Allele-specific genetic interactions of *GIM1/YKE2* were also observed with *SPC97*: deletion of *GIM1/YKE2* was synthetically toxic in *spc97-20* cells, while it did not affect growth of the *spc97-14* mutant (Figure 4C). In contrast to Δ*gim1/yke2*, Δ*gim2/pac10* or Δ*gim4* combined with *spc98-1*, *spc98-2* or *spc97-20* hardly affected growth (Table II). It is important to note that the *spc98* and *spc97* alleles have distinct phenotypes and are suppressed differently by *TUB4* (Geissler *et al.*, 1996; Knop *et al.*, 1997). In particular, *spc97-20* has an SPB duplication defect, while *spc97-14* fails to form a mitotic spindle (Knop *et al.*, 1997).

Finally, *GIM1/YKE2*, *GIM2/PAC10* and *GIM4* were in part tested for their genetic interactions with *TUB1*, *TUB2*, *BIN2*, *BIN3* and *RBL2*. *TUB1* and *TUB2* code for α- and β-tubulin in yeast (Neff *et al.*, 1983; Schatz *et al.*, 1986a), while *RBL2* encodes a β-tubulin-binding protein, the homologue of mammalian cofactor A (Archer *et al.*, 1995; Tian *et al.*, 1996). Bin2p and Bin3p are subunits of TRiC (Chen *et al.*, 1994). Δ*gim1* showed synthetic lethality with *tub1-4*, *tub2-403*, *tub2-405*, *bin2-1*, *bin3-1* and Δ*rbl2* (Table II). In contrast, Δ*gim4* was only synthetically lethal in combination with *bin2-1*, *bin3-1* and Δ*rbl2*, but not with *tub2-403* or *tub2-405*. In summary, the *GIM* genes show a broad range of genetic interactions with genes involved in tubulin biogenesis and microtubule nucleation.

Deletion of the GIM genes results in reduced levels of α-tubulin, which explains the benomyl super-sensitivity, but not the synthetic lethality with tub4-1

A defect of the *gim* null mutants in tubulin biogenesis may result in reduced levels of α-, β- or γ-tubulin. The amount of these proteins was determined by immunoblotting using specific antibodies. While the α-tubulin content was about half to a third compared with wild-type, β-tubulin and Tub4p levels were approximately the same (Figure 5A and B). It is noteworthy that deletion of *GIM4* affected α-tubulin less severely than deletion of the other *GIM* genes. We also noticed that the reduction of α-tubulin levels was independent of the incubation temperature of the cultures (data not shown).

An increase of β-tubulin over α-tubulin levels, which is the case in the *gim* null mutants, is toxic for yeast cells (Burke *et al.*, 1989; Katz *et al.*, 1990; Schatz *et al.*, 1986b). Therefore, increasing the α-tubulin content by overexpression of *TUB1* or reducing the pool of free β-tubulin by overproduction of the β-tubulin-binding protein Rbl2p may suppress the tubulin defects. Indeed, the benomyl super-sensitivity of the five Δ*gim* mutants was fully rescued when *TUB1* or *RBL2* were overexpressed using either elevated gene dosage (2μ plasmids) or the strong *ADH* promoter (Table I), while overexpression of

Table II. Genetic interactions of *GIM1/YKE2, GIM2/PAC10, GIM3, GIM4* and *GIM5* with genes involved in microtubule assembly

	tub4-1	*spc98-1/2*	*spc97-20*	*tub1-4*	*tub2-403/-405*	*bin2-1*	*bin3-1*	*Δrbl2*
gim1/yke2	SL[a]	SL	SL	SL	SL	SL	SL	SL
Δgim2/pac10	SL	+[b]	+	n.d.[c]	n.d.	n.d.	n.d.	SL
Δgim3	SL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	SL
Δgim4	SL	+	+	n.d.	+	SL[d]	SL	n.d.
Δgim5	SL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a]SL, synthetic lethality. This was determined by plasmid shuffle unless otherwise indicated. For plasmid shuffle experiments, the mutant strains (see Table III) were transformed with a plasmid-encoded *GIM* gene (*URA* plasmid). The corresponding *GIM* gene on the chromosome was disrupted by use of the *kanMX4* marker as described. The resulting strains were transformed with plasmids containing the *GIM* gene or the wild-type allele of the respective mutated gene and a control vector and were tested for growth on 5-FOA selecting against the *URA3* plasmid. Non-growth of the transformants with the control plasmid on 5-FOA indicates synthetic lethality. However, the transformants with the additional *GIM* gene or the wild-type allele of the mutated gene grew.
[b]+, not synthetically lethal.
[c]n.d., not determined.
[d]Tested for synthetic lethality by tetrad analysis. The *bin2-1* mutant and the *gim4* null mutant were crossed. The resulting strain was sporulated and the spores were analysed.

TUB4 showed no effect (Figure 5C; shown for *Δgim1/yke2*). We then investigated whether the reduced level of α-tubulin is also the cause for the synthetically lethal phenotype with *tub4-1*. However, overexpression of either *TUB1* or *RBL2* did not suppress the synthetic lethality, while plasmid-encoded *TUB4* and the *GIM* genes complemented the mutants (Figure 5D; shown for *Δgim1/yke2*). In summary, the benomyl super-sensitivity of the *GIM* mutants is the result of a reduced level of α-tubulin; however, this is not the reason for the synthetically lethal phenotype with *tub4-1*. This suggests that the Gim proteins have at least two functions, one related to α-tubulin and the other to Tub4p.

Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim5p bind to overproduced Tub4p

The failure of overexpressed *TUB1* and *RBL2* to suppress the synthetically lethal phenotype with *tub4-1* and the allele-specific interaction of *GIM1/YKE2* with *SPC98* and *SPC97* point to a more direct role for the Gim proteins in Tub4p complex function. This notion was supported further by the finding that overexpression of *TUB4* was toxic in the *Δgim1/yke2* null strain (Figure 6A, sector 1), while it hardly affected growth of the wild-type (Figure 6A, sector 3) (Spang *et al.*, 1996; Knop *et al.*, 1997). *Δgim1/yke2* cells overexpressing *TUB4* frequently were large-budded and showed abnormal bud morphologies (Figure 6A), phenotypes which were observed neither in *Δgim1/yke2* cells nor in wild-type cells overexpressing *TUB4* (data not shown). Similarly, simultaneous overexpression of chromosomally integrated *TUB4, SPC98* and *SPC97* was not toxic for wild-type cells; however, it strongly affected growth of the *gim1/yke2* null mutant (Figure 6B). As expected, this defect was rescued by plasmid-encoded Gim1p.

This result, together with the observed synthetically lethal phenotype with *tub4-1*, suggests that the *GIM* genes become essential either after overexpression of *TUB4* or in the presence of a mutated *TUB4*. This consideration led us to test whether the Gim proteins bind to overproduced Tub4p. A gene fusion consisting of *TUB4* and three repeats of the haemagglutinin (HA) epitope-encoding sequence was used for this experiment. *TUB4-3HA* was overexpressed in wild-type cells (Figure 6C), followed by the immunoprecipitation of Tub4p-3HA using anti-HA antibodies. Interestingly, the precipitate not only contained Tub4p-3HA (lane 3), but also Gim1p/Yke2p (lane 5), Gim2p/Pac10p (lane 7), Gim3p (lane 9) and Gim5p (lane 11). This co-immunoprecipitation was specific as no, or hardly any, Gim protein was detected when the anti-HA precipitation was performed with an extract containing overproduced Tub4p (lanes 6, 8, 10 and 12) instead of Tub4p-3HA. Gim4p was not tested due to the lack of an antibody. In summary, our results show that *GIM1/YKE2* becomes essential after *TUB4* overexpression and that the Gim proteins bind to overproduced Tub4p.

The Gim proteins interact with each other and are present in common complexes

The interactions between the Gim proteins were investigated by immunoprecipitation experiments, using functional, epitope-tagged versions of the Gim proteins (Figure 7A). For example, co-immunoprecipitation of Gim2p/Pac10p-MYC with Gim1p/Yke2p-3HA was observed. Similarly, Gim3p-MYC co-precipitated with Gim2p/Pac10p-3HA and Gim4p-3HA precipitated Gim2p/Pac10p-MYC. The result of the immunoprecipitation was confirmed using a functional gene fusion between *GIM5* and protein A (*GIM5-ProA*). Gim5p-ProA was enriched from cell lysates with IgG–Sepharose. We detected not only Gim5p-ProA in the precipitate, but also Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim4p-3HA (Figure 7B). Similar results were obtained with Gim2p/Pac10p-ProA (data not shown). Multiple interactions between the Gim proteins were also observed using the yeast two-hybrid system (Fields and Song, 1989) (data not shown).

The results of the immunoprecipitations do not allow a decision to be made as to whether the Gim proteins form only one or several complexes. To address this point, yeast extract from wild-type cells was fractionated by gel filtration (Figure 7C). Immunodetection of the Gim proteins revealed that Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim5p co-fractionated over a wide range, suggesting that the complexes were heterogeneous in their composition. However, all four proteins peaked in the same fraction corresponding to an apparent mol. wt of ~170–230 kDa. It is noteworthy that Gim2p/Pac10p was also detected in earlier fractions corresponding to mol.

wts up to 2 MDa. In conclusion, the immunoprecipitation experiments demonstrate that the Gim proteins interact with each other and that they are present in common complexes.

The Gim complexes are localized mainly in the cytoplasm of yeast cells

In order to obtain a better understanding of the Gim complexes, we investigated their cellular localization. Localization studies by indirect or direct immunofluorescence using epitope-tagged or green fluorescent protein (GFP) fusion proteins excluded a localization of the Gim proteins at the SPB. Instead, a cytoplasmic staining was observed (data not shown). This result was confirmed by fractionation experiments (Figure 8). Yeast cell lysates (lane 1) were separated by differential centrifugation into a soluble cytoplasmic fraction (lane 2) and a sediment (lane 3) containing nuclei, mitochondria and other organelles. The distribution of the α- and β-subunits of the cytoplasmic fatty acid synthase (Fas) and the nuclear protein nucleolin (Nop1p) established that the two fractions were hardly contaminated by each other. Gim1p/Yke2p, Gim2p/Pac10p, Gim3p, Gim4p-3HA and Gim5p were detected predominantly in the cytoplasmic fraction (lane 2). Taken together, the localization studies suggest that the Gim complexes are localized predominantly in the cytoplasm of yeast cells.

The mammalian homologues of Gim1p/Yke2p and Gim2p/Pac10p are fully functional in yeast

Gim1p/Yke2p is 47% identical to the mouse *KE2* gene product (Shang *et al.*, 1994) and Gim2p/Pac10p is 43% identical to the human VBP-1 protein (Geiser *et al.*, 1997). We were interested to see whether the *KE2* and *VBP-1* gene products can substitute for Gim1p/Yke2p and Gim2p/Pac10p in yeast. The *KE2* and *VBP-1* genes were amplified by PCR from a mouse and a human cDNA library, respectively. Both genes were cloned behind the yeast *ADH* promoter. Like plasmid-encoded *GIM1/YKE2*, the *ADH–KE2* promoter fusion complemented the synthetically lethal phenotype of Δ*gim1/yke2 tub4-1* (data not shown) as well as the cold- and benomyl-sensitive growth defects of Δ*gim1/yke2* cells (Figure 9A). Similar results were observed with the human *VBP-1* gene using the Δ*gim2/pac10* mutant (Figure 9B). Our results indicate that the Gim proteins are not only structurally conserved, but that they also fulfil very similar functions in mammalian and in yeast cells.

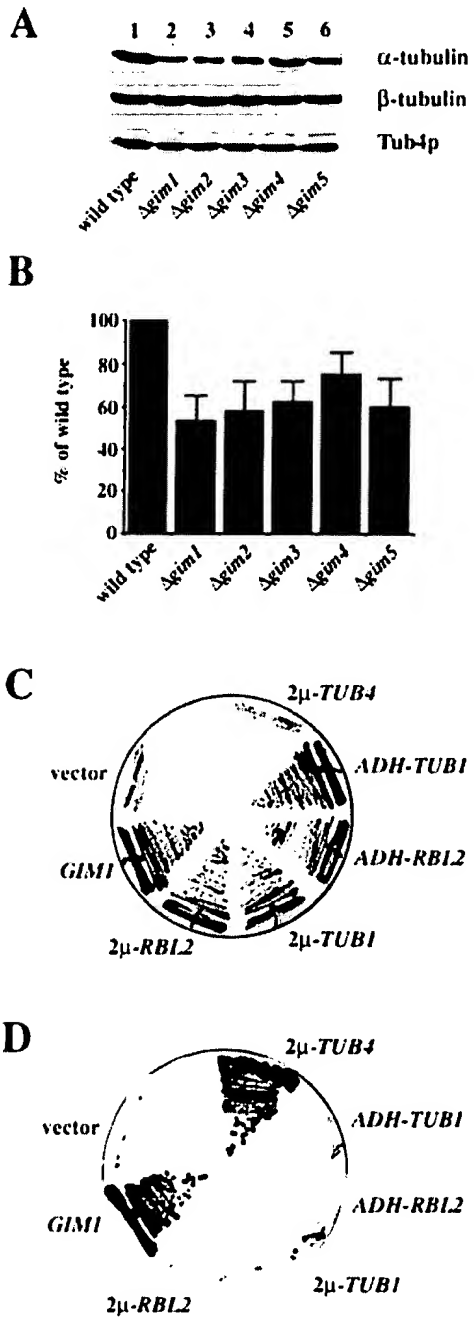


Fig. 5. Haploid *gim* null mutants have reduced α-tubulin levels, resulting in benomyl super-sensitivity. (**A**) Wild-type cells (lane 1) and cells of Δ*gim1/yke2* (lane 2), Δ*gim2/pac10* (lane 3), Δ*gim3* (lane 4), Δ*gim4* (lane 5) and Δ*gim5* (lane 6) were grown in YPD medium at 30°C to mid-log phase. Equal amounts of protein (50 μg) from the six strains were separated by SDS–PAGE, followed by immunodetection of α-tubulin, β-tubulin and Tub4p, using specific antibodies. An identical result was obtained when the cells were incubated at 14°C for 24 h (data not shown). (**B**) The α-tubulin levels of the wild-type and the five *gim* null strains from three independent experiments were quantified by densitometric scanning of the blots using the NIH image program. The bars indicate the variation between the experiments. (**C**) Cells of Δ*gim1/yke2* were transformed with control vector pRS425 (sector: vector), pRS315-*GIM1/YKE2* (*GIM1*), pRS425-*RBL2* (2μ-*RBL2*), pRS425-*TUB1* (2μ-*TUB1*), p415-*ADH-RBL2* (*ADH-RBL2*), p415-*ADH-TUB1* (*ADH-TUB1*) and YEp13-*TUB4* (2μ-*TUB4*). Transformants were tested for growth on YPD plates containing 2.5 μg/ml benomyl at 30°C. (**D**) In a similar manner as in (C), cells of strain SGY146 (*tub4-1* Δ*gim1/yke2* pRS316-*TUB4*) were transformed with the indicated plasmids. The transformants were tested for growth on 5-FOA plates at 30°C. Cells of SGY146 with the control vector pRS425 did not grow on 5-FOA, since *tub4-1* is synthetically lethal with Δ*gim1/yke2*. The synthetic lethality was relieved by an additional *TUB4* or *GIM1/YKE2* on a *LEU2*-based plasmid. In contrast to (C), *TUB1* or *RBL2* on pRS425 or under the control of the *ADH* promoter did not suppress the synthetic lethality of Δ*gim1/yke2 tub4-1*, indicated by the non-growth on 5-FOA.

Discussion

Gim1p/Yke2p, Gim2p/Pac10p, Gim3p, Gim4p and Gim5p interact with each other and form complexes

In this study, we performed a genetic screen for mutants that are not viable in combination with a mutated *TUB4*. We expected to find genes coding either for components of the Tub4p complex or for proteins that are involved in the folding of Tub4p or the assembly of the Tub4p complex. Since the Tub4p complex is associated with SPB substructures that are located in the nucleus (inner plaque) and in the cytoplasm (outer plaque) (Rout and Kilmartin, 1990; Spang *et al.*, 1996; Knop *et al.*, 1997), we could also expect to identify factors that play a role in the nuclear import of the Tub4p complex (G.Pereira *et al.*, 1998). Finally, we expected to find either α- or β-tubulin, since it has been proposed that these proteins interact with γ-tubulin (Oakley, 1992).

Our genetic screen identified *SPC98* and *SPC97*, which was not surprising, since previous results indicated that they interact genetically with *TUB4* (Geissler *et al.*, 1996; Knop *et al.*, 1997). In addition, we identified five novel genes that we named *GIM1–GIM5*. Two of the *GIM* genes have been described previously: *YKE2* was analysed due to its homology to the mouse *KE2* gene, located on the mouse MHC locus (Shang *et al.*, 1994), and *PAC10* was found in a screen for genes which become essential in the absence of *CIN8*, encoding a kinesin-related protein (Geiser *et al.*, 1997). The co-immunoprecipitation and the fractionation studies indicate that all five Gim proteins function together and that they are part of either one complex or several complexes containing different Gim proteins and possibly as yet unidentified proteins. In retrospect, our screen was quite selective, as it only identified components of the Tub4p or the Gim complexes.

Gim proteins have multiple functions related to the yeast cytoskeleton

All *GIM* deletion mutants had a reduced level of α-tubulin, while the amounts of β-tubulin and Tub4p were normal. The imbalance of α- and β-tubulin results in an elevated pool of free β-tubulin which is probably the cause of the microtubule defects. Our conclusion is supported by the observation that the deletion of *TUB3*, coding for the minor α-tubulin in yeast, also results in benomyl super-sensitivity (Schatz *et al.*, 1986b) and that overexpression of *TUB2*, coding for β-tubulin, is toxic (Katz *et al.*, 1990). We reasoned, that any manipulation that increases the amount of α-tubulin or decreases the pool of free β-tubulin should suppress the tubulin defects. This was indeed the case, as overexpression of *TUB1* or *RBL2*, both of which reduce the level of free β-tubulin (Archer *et al.*, 1995), cured the benomyl super-sensitivity of the *gim* null strains. We also believe that the synthetically lethal defects of *GIM* deletions with Δ*rbl2* are the result of too little α-tubulin, as loss of Rbl2p will increase the pool of free β-tubulin further, which is then lethal for the cells (Katz

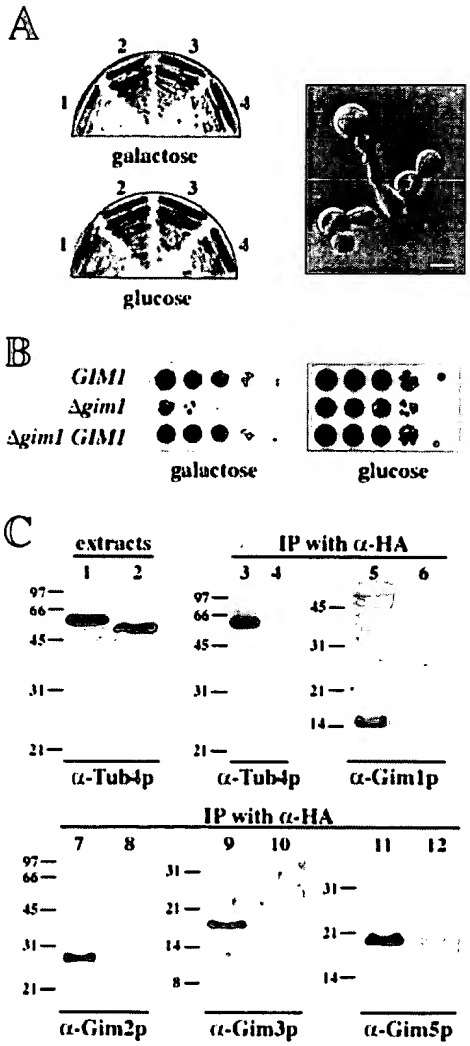


Fig. 6. Genetic interactions of *GIM1/YKE2* with *TUB4* and binding of the Gim proteins to overproduced Tub4p. (**A**) Overexpression of *TUB4* is toxic for Δ*gim1/yke2* cells. Cells of Δ*gim1/yke2* (sectors 1 and 2; strain SGY101) or *GIM1/YKE2* (sectors 3 and 4; strain YPH499) with plasmids pYES2-*TUB4* (*Gal1-TUB4*) (sectors 1 and 3) or pYES2 (sectors 2 and 4) were grown at 30°C on plates containing either raffinose/galactose (induction of the *Gal1* promoter) or glucose (no induction) as carbon sources. Δ*gim1/yke2 Gal1-TUB4* cells grown for 8 h in raffinose/galactose medium at 30°C were inspected by light microscopy. This culture contained a high proportion of cells with a large bud (>70%), and ~5% with a misformed bud. In contrast, 30–40% of the *GIM1* cells overexpressing *TUB4* contained a large bud, and <0.1% of the buds were misformed. Bar: 5 μm. (**B**) Cells co-overexpressing *TUB4*, *SPC98* and *SPC97* require *GIM1/YKE2* for survival. Serial dilutions of cells containing chromosomally integrated *Gal1-TUB4*, *Gal1-SPC98* and *Gal1-SPC97* in either a *GIM1/YKE2* (strain ESM387) or a Δ*gim1/yke2* (strain SGY159) background and cells of SGY159 transformed with plasmid-encoded *GIM1/YKE2* were grown on plates containing either glucose (no induction) or raffinose/galactose (induction) as carbon sources at 30°C. (**C**) Co-immunoprecipitation of Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim5p with overproduced Tub4p-3HA. YPH499 cells carrying plasmid-encoded *Gal1-TUB4-3HA* (pSI57) (lanes 1, 3, 5, 7, 9 and 11) or *Gal1-TUB4* (pSM209) (lanes 2, 4, 6, 8, 10 and 12) were grown in raffinose to a cell density of 5×10^6 cells/ml at 30°C. The *Gal1* promoter was induced for 6 h by the addition of galactose (2%). Cell extracts (lanes 1 and 2) were analysed by immunoblotting with anti-Tub4p antibodies, in order to confirm *TUB4-3HA* and *TUB4* overexpression. Cell lysates from both strains were incubated with anti-HA antibodies covalently coupled to protein A–Sepharose. The immunoprecipitates were analysed for Tub4p (lanes 3 and 4), Gim1p/Yke2p (lanes 5 and 6), Gim2p/Pac10p (lanes 7 and 8), Gim3p (lanes 9 and 10) and Gim5p (lanes 11 and 12) by immunoblotting using affinity-purified antibodies.

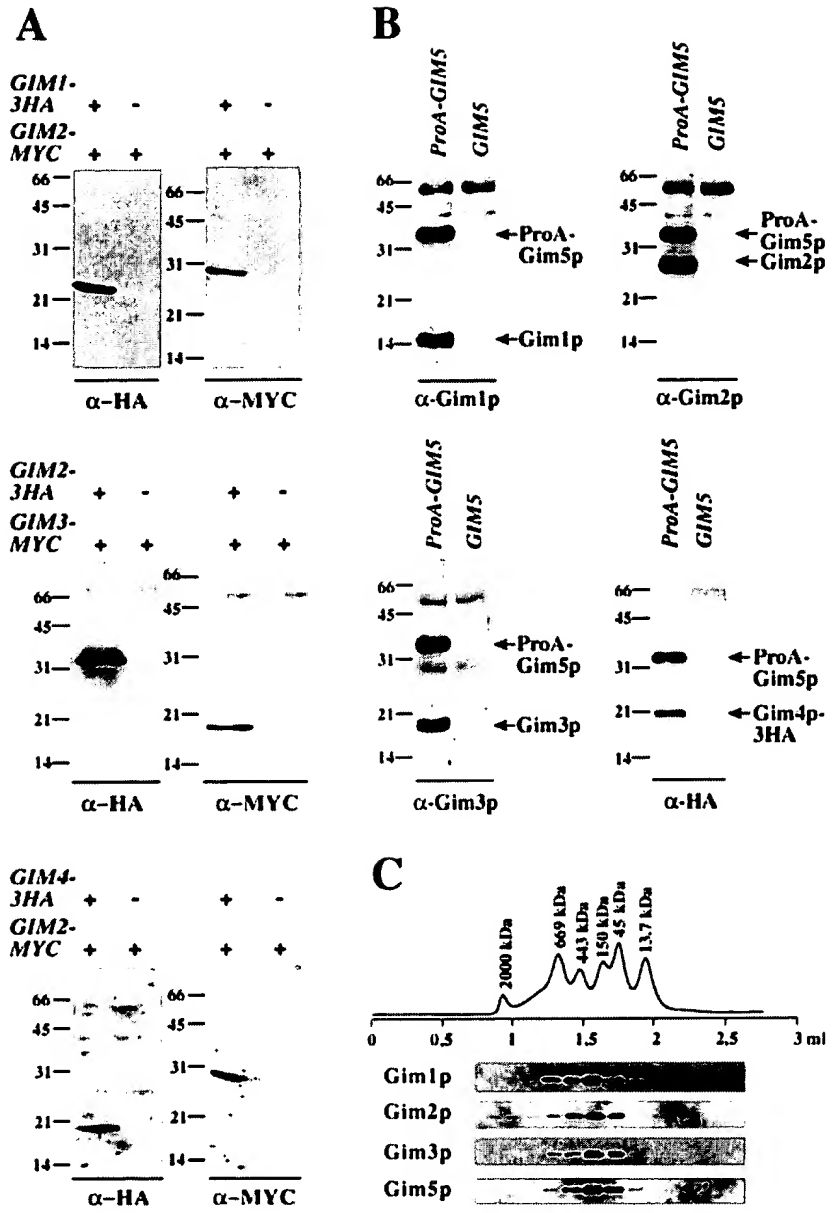


Fig. 7. Co-immunoprecipitation and co-fractionation of the Gim proteins. (A) Co-immunoprecipitation of the Gim proteins. Precipitation of the HA-tagged proteins of strains expressing the indicated *HA* and *MYC* gene fusions, or the wild-type genes, was performed with anti-HA antibodies coupled to protein A–Sepharose. Proteins were detected by immunoblotting using anti-HA and anti-MYC antibodies. (B) Co-immunoprecipitation of Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim4p-3HA with Gim5p-ProA. A chromosomally integrated gene fusion of *GIM5* with protein A was created. Cell lysates of ProA-*GIM5* or *GIM5* cells were incubated with IgG–Sepharose. The precipitates were analysed for Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim4p-3HA by immunoblotting using polyclonal anti-Gim1p/Yke2p, anti-Gim2p/Pac10p and anti-Gim3p antibodies, or monoclonal anti-HA antibodies. Gim5p-ProA was visualized by the binding of IgG–peroxidase to the ProA region of Gim5p-ProA. (C) The Gim proteins co-fractionate in gel filtration experiments. Cell lysates of strain YPH499 were fractionated by gel filtration. Fractions were analysed by immunoblotting using antibodies directed against the indicated proteins. The column was equilibrated using the indicated molecular weight standards.

et al., 1990; Archer *et al.*, 1995). Furthermore, the reduced level of α-tubulin in Δ*pac10* cells may explain why this gene becomes essential in the absence of the microtubule-binding protein Cin8p (Geiser *et al.*, 1997). Alternatively, Pac10p may have additional, motor protein-related functions.

GIM deletion mutants are slightly more sensitive towards the actin inhibitor latrunculin-A, than *tcp1-1* which codes for a mutated subunit of TRiC. Besides having other functions, the latter plays an essential role in actin folding (Ursic and Culbertson, 1991; Chen *et al.*, 1994; Miklos *et al.*, 1994; Vinh and Drubin, 1994). An additional hint of a function for the Gim proteins in actin-related processes comes from the sensitivity of *GIM* deletion mutants towards high osmolarity, which is also a common phenotype of actin mutants (Drubin, 1990). Since we did not observe direct defects of the actin cytoskeleton in *gim* null mutants and since latrunculin-A is highly specific towards actin (Ayscough *et al.*, 1997), the actin defects in the *gim* strains are probably only subtle.

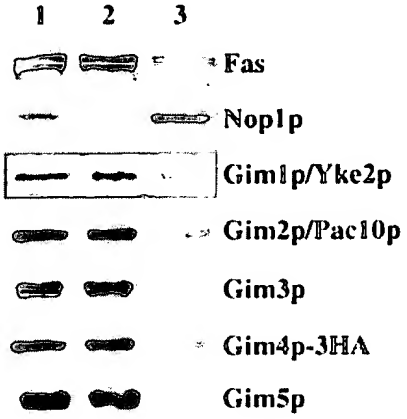


Fig. 8. The Gim complexes are localized mainly in the cytoplasm of yeast cells. Total cell lysates (lane 1) of wild-type (YPH499) and *GIM4-3HA* cells (ESM464) were fractionated by differential centrifugation (Young and Tyk, 1997) into a cytoplasmic 100 000 *g* supernatant (lane 2) and an organelle-containing pellet (lane 3). The sediment was resuspended in the original volume. Equal volumes were analysed by immunoblotting using antibodies directed against the indicated proteins.

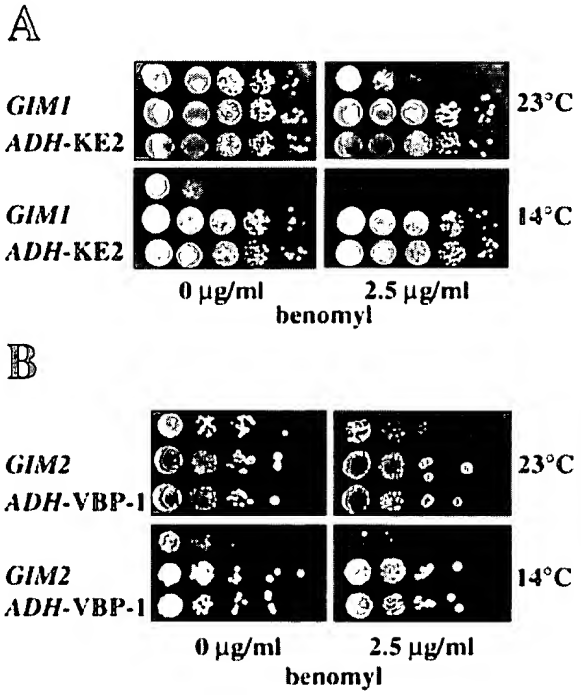


Fig. 9. The mouse *KE2* gene and the human *VBP-1* gene function for *GIM1/YKE2* and *GIM2/PAC10*, respectively, in yeast. (**A**) The cold sensitivity and the benomyl sensitivity of Δ*gim1/yke2* cells were complemented by expression of the *KE2* gene in yeast. Cells of Δ*gim1/yke2* were transformed with a control plasmid (p415-*ADH*, first row), *GIM1/YKE2* on plasmid pRS315 or a plasmid carrying the *KE2* gene under control of the yeast *ADH* promoter (p415-*ADH*-KE2). Serial dilutions of the transformants were tested for growth on YPD plates with or without 2.5 μg/ml benomyl at 23 or 14°C.
(**B**) Expression of VBP-1 rescues the cold-sensitive growth defect and the benomyl super-sensitivity of Δ*gim2/pac10* cells. Cells of Δ*gim2/pac10* were transformed with p415-*ADH* (first row), pRS315-*GIM2/PAC10* or p415-*ADH*-VBP-1. Transformants were tested as described in (A).

A number of results point to specific functions of the Gim proteins with respect to Tub4p. First, all *GIM* genes genetically interact with *TUB4*. Second, *GIM1/YKE2* shows allele-specific genetic interactions with *SPC98* and *SPC97*, coding for components of the yeast γ-tubulin complex. Third, overexpression of *TUB4* or co-overexpression of *TUB4*, *SPC98* and *SPC97* is toxic in a *gim1/yke2* null strain, while it does not affect growth of wild-type cells (Spang *et al.*, 1996; Knop and Schiebel, 1997). This suggests that the *GIM* genes become essential in the presence of a mutated Tub4p protein or after overproduction of Tub4p. Under the latter condition, we could show that the tested Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim5p proteins were associated with Tub4p.

Do all of the Gim proteins have the same function? The very similar phenotypes of the *gim* null strains as well as complex formation suggest that the Gim proteins have at least overlapping functions. However, we also obtained evidence for specialized functions of the Gim proteins. For example, only Δ*pac10* cells but not the other Δ*gim* mutants have a spore germination defect (Geiser *et al.*, 1997). In addition, Δ*gim4* cells have a less severe growth defect and are not as osmotically sensitive as the other *GIM* deletion mutants. Finally, *GIM1/YKE2*, *GIM2/PAC10* and *GIM4* interact differently with *SPC98* and *SPC97*.

The GIM genes encode phylogenetically conserved proteins which may function in folding or assembly processes

How do the Gim proteins function? The cellular localization, the effects of *gim* null mutants on the actin and tubulin systems as well as the phylogenetic conservation of the Gim proteins are reminiscent of the cytoplasmic chaperonin TRiC. Our localization studies suggests that the Gim proteins, like the TRiC proteins, form cytoplasmic multiprotein complexes. While TRiC contains eight subunits (Kubota *et al.*, 1995; Willison and Horwich, 1996; Nitsch *et al.*, 1997), it is still unclear whether additional Gim proteins exist. The tubulin defects of TRiC mutants and *gim* null strains are remarkably similar. TRiC mutants are also frequently super-sensitive towards benomyl, and their microtubules depolymerize at reduced temperatures. In addition, some of the TRiC mutants also do not arrest in the cell cycle, although microtubule structures are impaired (Ursic and Culbertson, 1991; Chen *et al.*, 1994; Miklos *et al.*, 1994). *tcp1-1* was similarly sensitive towards latrunculin-A as the *gim* null strains. Finally, a similar phylogenetic conservation as for the Gim proteins has been observed for the subunits of TRiC (Kubota *et al.*, 1995; Nitsch *et al.*, 1997): like TRiC, the Gim proteins have relatives in human, mouse, *C.elegans* and *S.pombe* (Figure 1). As for the Gim proteins, only two homologues of TRiC subunits were identified in archaebacteria and none in eubacteria. In addition to this, the Gim proteins as well as the subunits of TRiC bear a stronger homology to their relatives from other species than to each other (Kubota *et al.*, 1995; Waldmann *et al.*, 1995; Nitsch *et al.*, 1997).

The Gim complexes may play a role in the biogenesis of α- and γ-tubulin and probably actin, possibly as a molecular chaperone. A number of our results are consistent with this notion. First, the *GIM* genes show

Table III. Yeast strains and plasmids

Name	Genotype/construction	Source or reference
Yeast strain		
YPH499	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)
YPH500	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)
YPH501	*MATa/α ura3-52/ura3-52 lys2-801/ lys2-801 ade2-101/ade2-101trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1*	Sikorski and Hieter (1989)
239.2C	*MATa ade2-101 lys2-801 ura3-52 tub1-4*	T.Stearns
DBY1991	*MATα his4-619 act1-2*	D.Botstein
DBY1995	*MATα his4-619 act1-3*	D.Botstein
DBY2305	*MATa ura3-52 lys2-801 his4-539 tub2-403*	Schatz *et al.* (1988)
DBY2309	*MATa ura3-52 lys2-801 his4-539 tub2-405*	Schatz *et al.* (1988)
DDY805	*MATa ura3-52 his4-619 bin2-1*	Chen *et al.* (1994)
DDY806	*MATa his3Δ200 lys2-801 ura3-52 bin3-1*	Chen *et al.* (1994)
DUY559	*MATα leu2-3, -112 ura3-52 trp1-7 Δtcp1::LEU2 YCpMS38 (tcp1-1-TRP1)*	Ursic and Culbertson (1991)
ESM97	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δbub2::HIS3*	Spang *et al.* (1996)
ESM183	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δtub4::HIS3* pSM223	Spang *et al.* (1996)
ESM210	*MATa/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 TUB4/tub4-1*	Spang *et al.* (1996)
ESM243	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1* pSM296	Geissler *et al.* (1996)
ESM387	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 leu2Δ1::Gal1-TUB4-LEU2 trp1Δ63::Gal1-SPC97-3HA-TRP1 ura3-52::Gal1-SPC98-URA3*	Knop and Schiebel (1997)
ESM447	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 GIM5-ProA-kanMX4*	this study
ESM463	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 GIM4-3HA-kanMX4*	this study
K2346	*MATa ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$*	K.Nasmyth
K2348	*MATα ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$*	K.Nasmyth
KSY2	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim2/pac10::kanMX4*	this study
SGY24	*MATa ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$ Δtub4::HIS3* pSG24	this study
SGY33	*MATα ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$ Δtub4::HIS3* pSG24 pSG23	this study
SGY88	*MATa ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$ SPC98::LEU2-SPC98 Δtub4::HIS3* pSG24	this study
SGY101	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4*	this study
SGY110	*MATa/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 TUB4/tub4-1 Δgim4::HIS3MX6*	this study
SGY115	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim4::HIS3MX6*	this study
SGY116	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δgim4::HIS3MX6*	this study
SGY117	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim3::kanMX4*	this study
SGY119	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δtub4::HIS3 Δgim1/yke2::kanMX4* pSM223	this study
SGY120	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc98::HIS3 Δgim1/yke2::kanMX4* pSM296	this study
SGY121	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3 Δgim1/yke2::kanMX4* pMK8	this study
SGY123	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δgim3::HIS3MX6*	this study
SGY124	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δgim2/pac10::HIS3MX6*	this study
SGY140	*MATa ura3-52 lys2-801 his4-539 tub2-403 Δgim1/yke2::kanMX4* pSG62	this study
SGY141	*MATa ura3-52 lys2-801 his4-539 tub2-405 Δgim1/yke2::kanMX4* pSG62	this study
SGY143	*MATa bin3-1 his3Δ200 lys2-801 ura3-52 Δgim1/yke2::kanMX4* pSG62	this study
SGY144	*MATa ade2-101 lys2-801 ura3-52 tub1-4 Δgim1/yke2::kanMX4* pSG62	this study
SGY146	*MATα lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tub4-1 Δgim1/yke2::kanMX4* pSM223	this study
SGY156	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim5::kanMX4*	this study
SGY158	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δrbl2::HIS3MX* pSG62	this study
SGY159	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 leu2Δ1::Gal1-TUB4-LEU2 trp1Δ63::Gal1-SPC97-3HA-TRP1 ura3-52::Gal1-SPC98-URA3 Δgim1/yke2::kanMX4*	this study
SGY172	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δgim5::HIS3MX6*	this study
YMK10	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3* pMK8	Knop *et al.* (1997)
Plasmids		
p415-ADH	*CEN6, LEU2*-based yeast–*E.coli* shuttle vector carrying the *ADH* promoter	Mumberg *et al.* (1995)
pCH1122	YCp50 with *ADE3* and mutated centromere sequence	Kranz and Holm (1990)
p2–7–1	YEp13 containing *TUB4*	Geissler *et al.* (1996)
pMK8	pRS316 containing *SPC97*	Knop *et al.* (1997)
pMK10	pRS315 containing *SPC97*	M.Knop
pSG23	pRS314 containing *tub4-1*	this study
pSG24	pCH1122 containing *TUB4*	this study
pSG55	pRS315 containing *GIM1/YKE2*	this study
pSG60	pRS413 containing *GIM1/YKE2*	this study
pSG62	pRS316 containing *GIM1/YKE2*	this study
pSG64	pRS315 containing *MYC-GIM1/YKE2*	this study
pSG72	p415-*ADH* containing the mouse *KE2* gene	this study

Table III. *Cont.*

Name	Genotype/construction	Source or reference
pSG89	pGEX-4T-3 containing *GIM1/YKE2*	this study
pSG96	pRS315 containing *3HA-GIM1*	this study
pSG100	p415-ADH containing *GIM4*	this study
pSI2	pRS316 containing *GIM2/PAC10*	this study
pSI11	pRS315 containing *MYC-GIM2/PAC10*	this study
pSI13	pRS316 containing *MYC-GIM3*	this study
pSI14	pRS315 containing *GIM3*	this study
pSI16	pRS316 containing *MYC-GIM2/PAC10*	this study
pSI18	pRS315 containing *GIM2/PAC10*	this study
pSI25	pGEX-5X-1 containing *GIM2/PAC10*	this study
pSI26	pGEX-5X-1 containing *GIM3*	this study
pSI39	pRS315 containing *3HA-GIM2/PAC10*	this study
pSI41	pRS316 containing *MYC-GIM3*	this study
pSI57	pYES2 containing *TUB4-3HA*	this study
p*spc97-14*	pRS315 containing *spc97-14*	Knop *et al.* (1997)
p*spc97-20*	pRS315 containing *spc97-20*	Knop *et al.* (1997)
pSM204	pRS315 containing *tub4-1*	Spang *et al.* (1996)
pSM209	pYES2 containing *TUB4*	Spang *et al.* (1996)
pSM223	pRS316 containing *TUB4*	Spang *et al.* (1996)
pSM291	pRS315 containing *SPC98*	Geissler *et al.* (1996)
pSM296	pRS316 containing *SPC98*	Geissler *et al.* (1996)
pSM473	p415-*ADH* containing the coding region of *GIM5*	this study
pSM480	pRS315 containing *3HA-GIM4*	this study
pSM495	p415-*ADH* containing human *VBP-1*	this study
pSM553	pRS425 containing *TUB1*	this study
pSM554	pRS425 containing *RBL2*	this study
pSM555	p415-*ADH* containing *TUB1*	this study
pSM556	p415-*ADH* containing *RBL2*	this study
p*spc98-1*	pRS315 containing *spc98-1*	Geissler *et al.* (1996)
p*spc98-2*	pRS315 containing *spc98-2*	Geissler *et al.* (1996)
pRS305	*LEU2*-integrating vector	Sikorski and Hieter (1989)
pRS315	*CEN6*, *LEU2*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS316	*CEN6*, *URA3*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS413	*CEN6*, *HIS3*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS425	2 μm, *LEU2*-based yeast–*E.coli* shuttle vector	Christianson *et al.* (1992)
pGEX-4T-3	*E.coli* expression vector containing GST under control of the *lacZ* promotor	Pharmacia
pGEX-5X-1	*E.coli* expression vector containing GST under control of the *lacZ* promotor	Pharmacia
pYES2	2 μm, *URA3*-based yeast–*E.coli* shuttle vector carrying the *Gal1* promoter	Invitrogen

genetic interactions with components of TRiC, which is expected if both complexes function in related processes. Secondly, the Gim proteins bind to overproduced Tub4p. Thirdly, the reduced level of α-tubulin may be the consequence of a folding defect. Fourthly, the phenotypes of TRiC and *GIM* mutants are very similar. The Gim complexes may assist protein folding either before or after TRiC function. They could also bind to folded monomeric Tub4p or actin, thereby reducing potential toxic effects of the monomers. In any case, the Gim proteins only become essential for growth under certain circumstances, such as in the presence of a mutated Tub4p protein or overexpressed Tub4p.

Our results demonstrate that the mammalian homologues of Gim1p/Yke2p and Gim2p/Pac10p, the *KE2* and *VBP-1* gene products, are functional in yeast. Their expression complemented the synthetically lethal defects of Δ*gim1/yke2* or Δ*gim2/pac10* with *tub4-1* as well as the benomyl super-sensitivity and cold sensitivity. Interestingly, *KE2* mRNA is most abundant in brain and testis (Abe *et al.*, 1988), tissues which have a high content of tubulin. It is expected that these tissues have a high level of proteins that are required for tubulin biogenesis, as is the case for TRiC (Silver *et al.*, 1979, 1987). The homologue of Gim2p/Pac10p, the VBP-1 protein, has been identified in a two-hybrid screen using the von Hippel–Lindau tumor suppressor gene as a bait (Tsuchiya *et al.*, 1996). Whether this interaction has any physiological significance remains to be determined. Homologues of Gim3p and Gim5p were also found; however, we have not yet tested whether they function in yeast. The fact that the KE2 and VBP-1 proteins can substitute for Gim1p/Yke2p and Gim2p/Pac10p in yeast raises the possibility that the homologues of the Gim proteins also form complexes that promote the formation of functional α- and γ-tubulin in mammals and other species.

Materials and methods

Media and general methods

Basic yeast methods and growth media were as described (Guthrie and Fink, 1991). For *Gal1*-controlled gene expression, yeast strains were grown in synthetic complete medium (SC) containing raffinose (2%) as carbon source. Glucose (2%) or galactose (2%) was added for the repression or induction of the *Gal1* promoter, respectively. Yeast strains were transformed by the lithium acetate method (Schiestl and Gietz, 1989). *Escherichia coli* strains were transformed by electroporation (Dower *et al.*, 1988). PCR was performed with Vent polymerase (New England Biolabs). Recombinant DNA methodology was performed as reported (Sambrook *et al.*, 1989).

Plasmids and yeast strains

Plasmids and yeast strains used and constructed during the course of this study are listed in Table III. The mouse *KE2* gene and the human

VBP-1 gene were amplified by PCR from a mouse and human cDNA library, respectively and the PCR products obtained were cloned into p415-*ADH*. Epitope-tagged versions of *GIM* genes were generated by recombinant PCR, cloned into pRS315 (Sikorski and Hieter, 1989) and tested for functionality in *gim* mutants. The coding regions of the intron containing the *GIM4* ORF were amplified by PCR from chromosomal yeast DNA and cloned into p415-*ADH* (Mumberg *et al.*, 1995). The intron of *GIM5* was removed by recombinant PCR. The coding region of *GIM5* was cloned into expression vector p415-*ADH*. Double deletion mutants of Δ*gim1/yke2* together with any one of Δ*gim2/pac10*, Δ*gim3*, Δ*gim4* or Δ*gim5* were generated by subsequent deletion of the respective ORFs using either *kanMX4* (Wach *et al.*, 1994) or *HIS3MX6* (Wach *et al.*, 1997) as selectable markers. Chromosomally integrated C-terminal fusions of the *GIM* genes with GFP, protein A and 3HA were generated by homologous recombination with PCR-amplified *GIM-GFP-kanMX4* (Wach *et al.*, 1997), *GIM-ProA-kanMX4* and *GIM-3HA-kanMX4* cassettes (E.Schiebel, unpublished). *TUB1* and *RBL2* were amplified by PCR and cloned into p415-*ADH* and pRS425 (Christianson *et al.*, 1992), respectively.

Isolation of mutants which are synthetically lethal with tub4-1

In order to identify mutants which are synthetically lethal with *tub4-1*, we used the *ade2/ade3* red/white colony sectoring system (Koshland *et al.*, 1985; Huffaker *et al.*, 1987). The haploid strain K2348 (*ade2 ade3*) was transformed with an *ADE3/URA3* plasmid containing *TUB4* (pSG24). This plasmid had a mutated centromere rendering it unstable under non-selective growth conditions. The chromosomal *TUB4* gene was disrupted by insertion of the *HIS3* marker using the Δ*tub4::HIS3* cassette of plasmid pSM219 (Spang *et al.*, 1996). This strain was transformed with a *TRP1*-based plasmid carrying the temperature-sensitive *tub4-1* allele (pSG23), yielding strain SGY33. The *tub4-1* allele harbours a single amino acid substitution which causes cells to die at 37°C while they stay alive at 30°C (Spang *et al.*, 1996). Strain SGY33 grows with red colony colour, since it is genotypic *ade2*, resulting in the accumulation of a red intermediate of the adenine biosynthetic pathway. Loss of the *ADE3* plasmid pSG24 under non-selective conditions changes the genotype from *ade2* to *ade2 ade3*, resulting in white or red/white sectoring colonies. Strain SGY33 was mutagenized with methane sulfonic acid ethyl ester (EMS, Sigma) to ~30% survival. Mutations that conferred synthetic lethality with *tub4-1* were identified on plates without selection for the plasmid pSG24 as red, non-sectoring colonies, since these cells depend on *TUB4* of the *ADE3*-containing plasmid. Among 60 000 screened colonies, 12 colonies with this phenotype were obtained. These were unable to grow on plates containing 5-fluoro-orotic acid (5-FOA), a drug that kills cells harbouring a *URA3* gene. They all regained the red/white sectoring phenotype and their ability to grow on 5-FOA when transformed with a *LEU2*-based plasmid containing the *TUB4* gene but not when transformed with a plasmid carrying *tub4-1*. Although all 12 mutants showed a red/white sectoring phenotype when transformed with an *SPC98*-containing plasmid, it was shown by crossings to strain SGY88 (*SPC98-LEU2*) and subsequent linkage analysis (see below) that the mutation causing synthetic lethality resided only in two cases in *SPC98*. In contrast, the red/white sectoring phenotype of the other mutants was due to suppression of the *tub4-1* phenotype by *SPC98* (Geissler *et al.*, 1996). The mutants were back-crossed twice to strain SGY24, which revealed that the phenotypes were caused by a single, recessive mutation. Crosses between the mutants identified seven complementation groups.

Identification and disruption of the GIM genes

Two of the mutants from our screen were complemented by *SPC97*, recently identified as the third component of the γ-tubulin complex in yeast (Knop *et al.*, 1997). The other eight mutants were transformed with DNA from a *LEU2/CEN*-based yeast genomic library (kindly provided by Dr K.Nasmyth), and the transformants were screened for regaining of the red/white sectoring phenotype. The genomic inserts of plasmids allowing cells to lose the *TUB4*-containing *ADE3/URA3* plasmid after re-isolation and re-transformation were sequenced. Subcloning and sequencing identified five genes which we named *GIM1–GIM5*.

The *GIM*, *SPC98* and *SPC97* genes on pRS305 (Sikorski and Hieter, 1989) were targeted to their chromosomal locations in strain SGY24. The resulting strains were then crossed with the corresponding mutants. Random spore analysis of the diploid strains revealed that, for example, *GIM1* was linked to the mutation conferring the synthetically lethal phenotype with *tub4-1*, since all sectoring haploid progenies did not contain the labelled *GIM1-LEU2*. The *GIM* genes were disrupted by insertion of the heterologous markers *kanMX4* (Wach *et al.*, 1994) or *HIS3MX6* (Wach *et al.*, 1997), resulting in the deletion of the entire ORFs. The *kanMX4* and *HIS3MX6* disruption cassettes were generated by PCR and transformed into diploid YPH501 or haploid YPH499 (in the case of *GIM2/PAC10)*. The deletion of the *GIM* genes in the resulting strains was confirmed by PCR.

Anti-Gim1p/Yke2p, anti-Gim2p/Pac10p, anti-Gim3p, anti-Gim5p antibodies, immunoblots, immunoprecipitation experiments and immunofluorescence microscopy

The entire coding regions of *GIM1/YKE2*, *GIM2/PAC10*, *GIM3* and *GIM5* were cloned into pGEX expression plasmids (Pharmacia). The GST fusion proteins were expressed and purified by affinity purification using glutathione–Sepharose according to the manufacturer's recommendations. Antibodies against the purified proteins were raised in rabbits as described (Harlow and Lane, 1988). Antibodies against Gim1p/Yke2p and Gim2p/Pac10p were affinity purified as described (Spang *et al.*, 1995). The specificity of the antibodies was demonstrated by performing immunoblots with extracts from *GIM* and Δ*gim* strains.

For immunoprecipitations of the Gim proteins, cells of strains carrying 3HA- or MYC-tagged derivatives were grown in selective media at 30°C to a density of 2×10^7 cells/ml. Washed cells were resuspended in H-buffer [50 mM HEPES, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine, 10 μM leupeptin, 20 μg/ml chymostatin and 20 μg/ml E64] and lysed by vortexing with glass beads on ice until >95% of the cells were lysed. Cell debris were removed by centrifugation (5 min, 20 000 *g*). Cell lysates were incubated with anti-HA antibodies (12CA5) covalently bound to protein A–Sepharose for 3 h at 4°C. The anti-HA antibodies were cross-linked to protein A–Sepharose as reported (Harlow and Lane, 1988). The precipitates were washed twice with H-buffer, resuspended in HU-buffer (Knop *et al.*, 1996) and heated for 10 min at 65°C prior to SDS–PAGE (Laemmli, 1970). Gim5p-ProA was precipitated identically using IgG–Sepharose. As molecular weight standards for SDS–PAGE, we used myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa).

For the overexpression of *TUB4-3HA* and *TUB4* from the *Gal1* promoter, yeast cells were grown in SC medium with 2% raffinose as sole carbon source at 30°C to a cell density of 5×10^6 cells/ml. The *Gal1* promoter was induced by the addition of 2% galactose for 6 h. Harvested cells were resuspended in L-buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 5% glycerol, 1 mM GTP and protease inhibitors) and lysed with glass beads as described above. Cell lysates were incubated with anti-HA antibodies covalently bound to protein A–Sepharose for 1 h at 4°C.

For the quantifications of α-tubulin, β-tubulin and Tub4p, cells were lysed as described by Knop *et al.* (1996). The primary antibodies used for immunoblots were mouse monoclonal anti-HA (12CA5, Hiss Diagnostics), mouse monoclonal anti-MYC (9E10; Boehringer Ingelheim), rabbit anti-Gim1p/Yke2p, rabbit anti-Gim2p/Pac10p, rabbit anti-Gim3p, rabbit anti-Gim5p, rabbit anti-Tub4p (Geissler *et al.*, 1996), rabbit anti-α-tubulin, rabbit anti-β-tubulin (α- and β-tubulin antibodies were a gift of Dr F.Solomon), rabbit anti-Nop1p (Dr E.Hurt) and rabbit anti-Fas antibodies (Dr R.Egner). As secondary antibodies, rabbit anti-mouse or goat anti-rabbit IgGs coupled to horseradish peroxidase (Jackson Immuno Research Laboratories) were used. The immunoreaction was visualized by an ECL Kit from Amersham.

Immunofluorescence of formaldehyde-fixed yeast cells was performed as described (Knop *et al.*, 1996) with 1 h fixation time. The primary antibodies were mouse monoclonal anti-β-tubulin WA3 (kindly provided by U.Euteneuer-Schliwa) or mouse monoclonal anti-actin (Amersham). Secondary antibodies were goat anti-mouse IgGs coupled to CY3 (Jackson Immuno Research Laboratories). DNA was stained with DAPI (Boehringer Mannheim).

Nocodazole arrest, flow cytometry, determination of cell size and protein determination

For nocodazole arrest, cells were treated with 15 μg/ml nocodazole (Sigma) in liquid medium at 30°C for 3 h. For flow cytometry, cells were prepared as reported (Hutter and Eipel, 1979). The DNA content of 20 000 cells was determined using a flow cytometer (Facscalibur, Becton-Dickinson). Mean cell volumes were determined using a CASY1 Cell Counter and Analyzer System (Schärfe System GmbH). Protein concentrations were determined according to Bradford (1976).

Size exclusion chromatography

Cell lysates of YPH499 were prepared in H-buffer as described above. Proteins were separated according to their apparent size using a Smart System from Pharmacia with a Superose 6 column (PC 3.2/30). The column was equilibrated with H-buffer, and molecular weight determination was carried out according to a calibration curve obtained with standard proteins (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, bovine serum albumin, ovalbumin, carbonic anhydrase, RNase A, blue dextran; Sigma) separated in H-buffer. Separation was performed with a flow rate of 15 μl/min. The eluate was collected in fractions of 50 μl each. Each three consecutive fractions were pooled and analysed by immunoblotting.

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Modulation of Tubulin Polypeptide Ratios by the Yeast Protein Pac10p

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ABSTRACT

Normal assembly and function of microtubules require maintenance of the proper levels of several proteins, including the tubulin polypeptides themselves. For example, in yeast a significant excess of β-tubulin causes rapid microtubule disassembly and subsequent cell death. Even the modest excess of β-tubulin produced by genetic alterations such as deletion of the minor α-tubulin gene *TUB3* affects cell growth and can confer microtubule phenotypes. We show here that the levels of the yeast protein Pac10p affect the relative levels of the tubulin polypeptides. Cells deleted for *PAC10* have the same phenotypes as do cells that express reduced levels of α-tubulin or Rbl2p, two proteins that bind β-tubulin. Conversely, overexpression of Pac10p enhances the ability of α-tubulin or Rbl2p to suppress the lethality associated with excess β-tubulin. However, Pac10p is itself not a β-tubulin binding protein. *Pac10* null cells show a 30% decrease in the ratio of α-tubulin to β-tubulin. The results suggest that Pac10p modulates the level of α-tubulin in the cell, and so influences microtubule morphogenesis and tubulin metabolism.

EARLY steps in the microtubule assembly pathway affect proper folding of the nascent tubulin chains and their incorporation into the heterodimer. Genetic and biochemical evidence demonstrates that folding of α- and β-tubulin is mediated by the Tcp-1p chaperone complex (Gao *et al.* 1992; Yaffe *et al.* 1992; Sternlicht *et al.* 1993). *In vitro*, other factors are essential, either to finish the folding reaction or to stabilize the tubulin chains until they are dimerized (Gao *et al.* 1993; Melki *et al.* 1996; Tian *et al.* 1996). In the budding yeast *Saccharomyces cerevisiae*, mutations in presumptive chaperone complex components affect microtubule assembly and function (Ursic and Culbertson 1991; Chen *et al.* 1994). However, these studies have not fully demonstrated how tubulin chains fold and assemble in cells.

The interactions of undimerized tubulin chains may have considerable physiological significance. In yeast, genetic configurations that produce higher than wild-type ratios of β- to α-tubulin are toxic (Burke *et al.* 1989; Katz *et al.* 1990; Weinstein and Solomon 1990). Acute overexpression of β-tubulin causes rapid, quantitative microtubule disassembly and subsequently a 10^4-fold decrease in cell viability. In contrast, overexpression of α-tubulin does not cause microtubule disassembly and is only slightly toxic (Weinstein and Solomon 1990). These different properties of α- and β-tubulin imply functional differences between the two proteins. For example, perhaps β-tubulin sequences are more important for interactions between the α-β tubulin heterodimer and factors essential for microtubule assembly than are α-tubulin sequences. Consequently, excess free β-tubulin could be an effective competitive inhibitor of assembly and thus toxic (Weinstein and Solomon 1992). The molecular targets of β-tubulin toxicity are as yet unidentified.

β-Tubulin lethality is efficiently suppressed by concomitant overexpression of α-tubulin, presumably by sequestering the excess β-tubulin in heterodimer. A screen for other genes that when overexpressed would also rescue β-tubulin lethality identified three *RBL* genes; one of them, *RBL2*, suppresses β-tubulin lethality as well as overexpressed α-tubulin (Archer *et al.* 1995). Like α-tubulin, Rbl2p binds specifically to β-tubulin. Cofactor A, a protein originally identified as part of an *in vitro* assay for β-tubulin folding (Campo *et al.* 1994; Gao *et al.* 1994; Tian *et al.* 1996), is structurally and functionally homologous to *RBL2* (Archer *et al.* 1995). Cofactor A is thought to bind to a relatively unfolded form of β-tubulin; however, *in vivo* Rbl2p can bind to β-tubulin both before and after it has been incorporated into heterodimer (Archer *et al.* 1998). Thus, the available *in vivo* evidence does not clearly define a role for Rbl2p in β-tubulin folding. Indeed, Rbl2p may participate in the formation of heterodimer or it may act as a buffer of free β-tubulin.

RBL2 is a nonessential gene. Δ*rbl2* strains do display modest microtubule phenotypes. We previously showed that some α-tubulin mutants require Rbl2p for viability. Because such genetic interactions can identify functions that are redundant with or act in conjunction with those of Rbl2p, we screened for mutations in genes other than α-tubulin that are synthetically lethal with Δ*rbl2*. Here we describe one such gene, *PAC10*. This gene was previously identified in a screen for genes that are required in the absence of *CIN8*, which encodes a microtubule

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motor protein (GEISER *et al.* 1997). We find that *pac10* null strains display phenotypes similar to those associated with deletion of *RBL2* (ARCHER *et al.* 1995) or of the minor α-tubulin gene *TUB3* (SCHATZ *et al.* 1986b). Unlike α-tubulin and Rbl2p, however, Pac10p does not form a complex with β-tubulin. Instead, our data suggest that *PAC10* expression levels affect the ratio of α-tubulin to β-tubulin, probably by modulating the level of α-tubulin. This effect explains the several microtubule-related phenotypes of altered Pac10p levels and suggests how Pac10p may function in early steps of microtubule morphogenesis.

MATERIALS AND METHODS

Strains and microbiological techniques: Genetic manipulations and growth media were standard methods (SHERMAN *et al.* 1986). The strains and plasmids used in this study are listed in Table 1.

Mutagenesis and mutant isolation: We mutagenized JAY551 (Δ*rbl2*) with ethylmethane sulfonate resulting in 40% viability. We plated 60,000 cells on media lacking uracil and replica plated to 5-fluoroorotic acid (5-FOA) to select for the ability to lose the plasmid pA21A bearing *RBL2*. After this first selection we isolated 105 candidates unable to grow on 5-FOA. The 5-FOA sensitivity of eight of those strains was rescued by transformation with plasmid pJA33 bearing *RBL2* marked with *HIS3*. The strains bearing the synthetic lethal mutation were backcrossed to a wild-type strain (FSY183) and segregants were tested for the mutant allele. To test allelism between the synthetic lethal mutations and α-tubulin, we crossed mutant strains to PAY60, a derivative of DBY2282 (provided by D. BOTSTEIN, Stanford University), in which the *TUB1* locus (linked to the *TUB3* locus) is marked with *LEU2*. If the synthetic lethal mutation is in one of the α-tubulin genes, its phenotype should segregate away from the *LEU* marker.

Immunological techniques: We followed standard procedures for immunoblots and immunofluorescence (SOLOMON *et al.* 1992), using anti-α-tubulin antibody #345 and anti-β-tubulin antibody #206 at a dilution of 1/3500 for the immunoblots; and antibody #206 at 1/2000 for immunofluorescence (WEINSTEIN and SOLOMON 1990).

Cloning of *RKS2/PAC10*: We used strain PAY3 (*rks2-1*) as a host to clone *RKS2*. We transformed these cells with a *S. cerevisiae* genomic DNA library on a centromeric plasmid marked with *URA3* (provided by C. THOMPSON and R. YOUNG, M.I.T.). We tested the 40,000 transformants for recovered resistance to 30 μg/ml benomyl and so identified 30 candidates. We isolated the suppressing plasmids from each, and characterized the inserts by restriction mapping and so identified a region common to all the inserts. Partial DNA sequencing of that region demonstrated that it was identical to *PAC10* [(GenBank accession no. U29137 (GEISER *et al.* 1997)]. A plasmid, pPA36, carrying a 1.4-kb *Bam*HI-*Kpn*I fragment that includes the entire *PAC10* gene was created by cutting pPA1 with *Bam*HI and *Kpn*I and religating this fragment into the backbone carrying *PAC10*.

Disruption of *PAC10* and *PAC2*: To disrupt the entire *PAC10* open reading frame (ORF), we used PCR to flank the *HIS3* gene with the 5′- and 3′-noncoding regions of *PAC10* (815 bp upstream of the initiation codon and 718 bp downstream of the termination codon). The PCR primers for the 5′-noncoding region were 5′-TCAGAAGGCAATGCTGAATC-3′ and 5′-AGATCTCCAAAGAAAAATAAAGGGCA-3′; and for the 3′-noncoding region, 5′-AGATCTATGTGCGTACAGTTTTC TGC-3′ and 5′-GCAGTGGTGATGATGATTGG-3′. The two fragments were cloned into the pGEM-vector (Promega, Madison, WI), generating the plasmid pPA10. The primers create a *Bam*HI site to permit cloning of a *Bam*HI fragment carrying the *HIS3* gene. This *PAC10::HIS3* fragment was cut from the plasmid and transformed into wild-type diploids (FSY185). We checked the transformants for the correct integration of the disruption fragment at the *PAC10* locus by PCR. A His^+ haploid containing the desired integration was backcrossed against wild-type cells and renamed strain PAY169 (Δ*pac10*).

A similar approach was used to disrupt the *PAC2* gene. The oligonucleotides 5′-TTCTTCTGGTGCAGTCAACG-3′ and 5′-GGATCCATCTCTGAAATTCGTTTTGC-3′) were used to generate a 1050-bp domain of the 5′ region; and GGATCCCCTTT TAGATTGTAAGCGGA-3′ and 5′-CAAAGACGGTAAACTAA AACAGCA-3′) were used to generate an 800-bp fragment of the 3′ region. A Δ*pac2* haploid was renamed as strain PAY175. The Δ*cin1* strain (JFY206) was provided by J. FLEMING.

Analysis of suppression of β-tubulin lethality: We transformed JAY47 with several combinations of *TUB1*, *RBL2*, and *PAC10* plasmids. To determine the extent of suppression of β-tubulin lethality, we plated the transformants to galactose (inducing) and glucose (noninducing) media. The extent of suppression is expressed as the percentage of cells growing on galactose *vs.* glucose media. We made an overexpression version of the *PAC10* gene by amplifying the coding region with PCR and cloning the fragment into the *Sal*I, *Not*I sites of a p*GAL-URA3-CEN* vector (LIU *et al.* 1992) to create pPA23 (*pGal-PAC10*). The primers for the *PAC10* gene were RKS2/*Sal*I: 5′-GTCGACTATGGACACACTGTTCAACTCCA-3′ and RKS2/*Not*I 5′-GCGGCCGCACAGACACATTATATCTTGAG-3′ creating pPA23 (p*GAL-RKS2*). The construction pPA23 was checked for its ability to rescue the benomyl supersensitivity of Δ*pac10* (PAY169) in a galactose-dependent manner. The other plasmids used in the experiment were pDK44 (*TUB1-LYS2-CEN*) and pJA33 (*RBL2-HIS3-CEN*).

Sensitivity to β-tubulin lethality: We made the diploid strain PAY224 by crossing PAY169 (Δ*rks2*) and FSY626 (*TUB2-LEU2-GalTUB2*), then sporulated to generate strains PAY231 (Δ*rks2*, *TUB2-LEU2-GalTUB2*) and PAY232 (*TUB2-LEU2-GalTUB2*). To test the effect of β-tubulin overexpression, PAY231 and PAY232 cells were grown overnight in raffinose media at 30°. At 0 hr, galactose was added to 2%, and at different time points samples were obtained. To test for viability, we counted the cells in each sample and plated them to glucose plates.

RESULTS

Identification of genes synthetic lethal with Δ*rbl2*: Cells lacking Rbl2p have conditional microtubule phenotypes, and Δ*rbl2* is synthetically lethal with specific mutant alleles of α-tubulin (ARCHER *et al.* 1995). The vertebrate homolog of Rbl2p, cofactor A, may be involved in folding nascent β-tubulin chains *in vitro* (GAO *et al.* 1994). To learn more about the cellular functions in which Rbl2p participates, we screened for new mutations that make this gene essential. We mutagenized a Δ*rbl2* strain bearing a *CEN* plasmid (pA21A) expressing genomic *RBL2* and the *URA3* marker (JAY551; see MATERIALS AND METHODS for details). This screen identified eight independent strains that require wild-type *RBL2* for growth. Each of the eight strains is benomyl supersensitive (Ben^s), two are cold sensitive (at 15°) and one is thermosensitive (at 37°). Backcrossing the

TABLE 1

Plasmids and strains

	Genotype	Reference
Strain		
JAY551	*MATα Δrbl2 ura3 leu2 his3 ade2* (pA21A)	ARCHER (1996)
FSY183	*MATa ura3 leu2 his3 lys2*	KATZ *et al.* (1990)
DBY2282	*MATα ura3 leu2 his3 lys2 trp1 TUB1-LEU2-TUB1*	SCHATZ *et al.* (1986a)
FSY185	*MATα/MATa ura3/ura3 leu2/leu2 his3/his3 lys2/lys2 ade2/ADE2*	KATZ *et al.* (1990)
JAY47	*MATα/MATa ura3/ura3 leu2/leu2 his3/his3 lys2/lys2 ade2/ADE2 TUB2/TUB2-LEU2-GAL TUB2*	ARCHER *et al.* (1995)
PAY1	*MATα Δrbl2 rks2-1 ura3 leu2 his3 ade2* (pA21A)	This article
PAY3	*MATα Δrbl2 rks2-1 ura3 leu2 his3 ade2* (pJA33)	This article
PAY60	*MATa ura3 leu2 his3 lys2 trp1 TUB1-LEU2-TUB1*	This article
PAY169	*MATa pac10:HIS3 ura3 leu2 his3*	This article
PAY175	*MATa pac2::HIS3 ura3 leu2 his3*	This article
PAY223	*MATα/MATa rks2-1/pac10::HIS3 ura3/ura3 his3/his3 leu2/leu2* (pA21A)	This article
PAY231	*MATa pac10::HIS3 ura3 leu2 his3 TUB2-LEU2-TUB2*	This article
PAY232	*MATa ura3 leu2 his3 TUB2-LEU2-TUB2*	This article
JAY528	*MATα rbl2::URA3 ura3 leu2 his3 lys2*	ARCHER (1996)
PAY170	*MATα pac10:HIS3 ura3 leu2 his3*	This article
JFY209	*MATa cin1:URA3 ura3 leu2 his3*	This article
Plasmid		
pA21A	*RBL2 URA3 CEN*	ARCHER *et al.* (1995)
pRB539	*TUB1 LEU2 CEN*	SCHATZ *et al.* (1986a)
pJA33	*RBL2 HIS3 CEN*	ARCHER (1996)
pDK44	*TUB1 LYS2 CEN*	KIRKPATRICK and SOLOMON (1994)
pRB624	*tub1-724 LEU2 CEN*	SCHATZ *et al.* (1988)
pRB628	*tub1-728 LEU2 CEN*	SCHATZ *et al.* (1988)
pRB638	*tub1-738 LEU2 CEN*	SCHATZ *et al.* (1988)
pRB659	*tub1-759 LEU2 CEN*	SCHATZ *et al.* (1988)
pRB614	*tub1-714 LEU2 CEN*	SCHATZ *et al.* (1988)
pRB627	*tub1-727 LEU2 CEN*	SCHATZ *et al.* (1988)
pRB630	*tub1-730 LEU2 CEN*	SCHATZ *et al.* (1988)
pRB646	*tub1-746 LEU2 CEN*	SCHATZ *et al.* (1988)
pPA1,2,3	*URA3 CEN* library plasmids containing *PAC10*	This article
pPA10	pGEM *pac10::HIS3*	This article
pPA12	pGEM *pac2::HIS3*	This article
pPA23	*GAL-PAC10 URA3 CEN*	This article
pPA36	*PAC10 URA3 CEN*	This article
pPA45	*GAL-PAC2 URA3 CEN*	This article
pPA46	*TUB3-CEN-URA3*	This article

mutagenized strains to wild-type cells (FSY183) demonstrates that these conditional phenotypes are recessive. Because *Δrbl2* is known to be synthetically lethal with specific α-tubulin mutations, we tested to determine if the double mutants could be rescued by excess α-tubulin. In all of the mutant strains, the presence of excess α-tubulin provided by genomic *TUB1* on a low-copy plasmid (pRB539) relieves the need for *RBL2*. In seven of the eight strains, the extra copy of *TUB1* also fully suppresses the Ben^s phenotype, as expected if the mutation were in either of the α-tubulin genes. However, in one of the strains—PAY1—we noticed that excess α-tubulin does not completely restore wild-type growth on benomyl, especially at higher concentrations (30–40 μg/ml) of the drug. In a direct test for allelism with α-tubulin, sporulation of the diploid resulting from crossing PAY1 with a strain bearing a *LEU2* marker integrated next to *TUB1* (PAY60) demonstrates that the benomyl supersensitivity segregates independently of the *LEU2* marker. Therefore, the new mutation is unlikely to reside in either α-tubulin gene *TUB1* or *TUB3*, which are themselves linked. We provisionally named the mutated locus *rks2-1* (*R*BL2 *K*nockout *S*ynthetic lethal).

Cloning of *RKS2*: To identify the wild-type *RKS2* sequence, we transformed PAY3 (*rks2-1*, *Δrbl2*, pJA33) with an *S. cerevisiae* genomic library marked with *URA3* and tested transformants for suppression of the benomyl supersensitivity. About 0.1% of the 4×10^4 transformants were able to grow on 30 μg/ml benomyl. Character-

ization of several of the suppressing plasmids demonstrated they contained three genomic fragments that shared a single domain. The overlapping region corresponds to a 600-bp ORF that predicts a 199aa protein of 23.1 kD. A mutation in this same sequence previously arose from a screen for genes synthetically lethal with deletion of the nonessential mitotic motor *CIN8* (GEISER *et al.* 1997). That report named the sequence *PAC10* and described two mutant alleles. Therefore, we renamed the *rks2* mutation from our screen *pac10-3*.

Phenotypes of *pac10* mutant cells: To characterize this presumptive *pac10* mutation further, and to establish that the synthetic lethal mutation is indeed allelic to *PAC10*, we removed the entire *PAC10* ORF by integrative transformation in the wild-type diploid strain FSY185. We used PCR to confirm the presence of one wild-type and one disrupted copy of *PAC10* in the resulting diploid (see MATERIALS AND METHODS). Sporulation of these heterozygotes produced tetrads containing primarily four viable spores, and the marker identifying the *pac10* disruption segregated 2:2. Thus Δ*pac10*, like *pac10-3*, is viable. We created a diploid strain, PAY223, designed to be heterozygous at the *PAC10* locus (Δ*pac10*/*pac10-3*), homozygous for Δ*rbl2*, and carrying wild-type *RBL2* on a low-copy plasmid marked with the *URA3* gene. Sporulation of this strain demonstrated that all segregants require the *RBL2* plasmid for viability. These results provide further evidence that *pac10-3* is indeed a mutant allele of *PAC10*. They also show that the null allele of *pac10*, like *pac10-3*, is synthetically lethal with Δ*rbl2*.

Analysis of Δ*pac10* cells demonstrates that they display the conditional phenotypes (Ben^s, moderate Cs$^-$ at 15°) of *pac10-3*. These phenotypes are similar to those displayed by cells containing a moderate excess of β-tubulin due to deletion of the minor α-tubulin gene, *TUB3* (SCHATZ *et al.* 1986b). Neither Δ*pac10* nor *pac10-3* has abnormal microtubules at either 30° or at 15°, as assessed by immunofluorescence.

Suppression of the Δ*pac10*, Δ*rbl2* synthetic lethality by mutant α-tubulins: The phenotypes associated with the *pac10* mutants—synthetic lethality with Δ*rbl2* and benomyl supersensitivity at modest (20 μg/ml) concentrations of benomyl—are largely suppressed by a low-copy plasmid bearing the major α-tubulin gene, *TUB1* (Figures 1 and 2). However, analysis of several cold-sensitive mutant α-tubulins shows that they vary in their ability to rescue these phenotypes. We transformed PAY189 cells (Δ*rbl2*, Δ*pac10*, p*CEN-RBL2-URA3*) with plasmids bearing *tub1* mutant alleles (SCHATZ *et al.* 1988). We assayed for the ability of these mutant genes to support growth in the absence of plasmid-borne wild-type *RBL2*. The *tub1* mutants we tested included representatives from each of the three classes originally described: those arresting with no microtubules (class 1), with too many microtubules (class 2), or with disorganized microtubules (class 3). We found that all of the

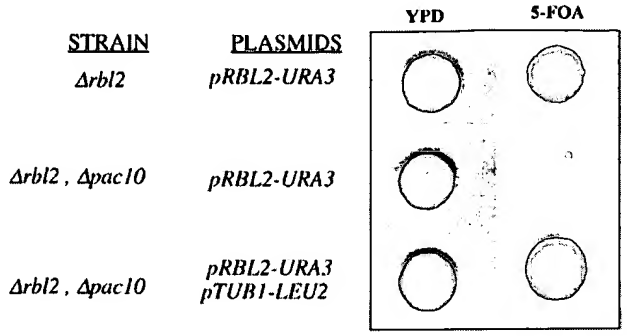

FIGURE 1.—The Δ*rbl2*, Δ*pac10* synthetic lethal interaction is rescued by overexpression of α-tubulin. Haploid cells bearing Δ*rbl2* and a *CEN* plasmid encoding *RBL2* and marked with the *URA3* gene (top row) can grow normally on YPD or on medium containing 5-FOA. Deletion in the same strain of the *PAC10* gene (middle row) causes these same cells to die on 5-FOA. This lethality is efficiently suppressed by the presence of a second plasmid encoding α-tubulin (third row).

mutant α-tubulins assayed can suppress the lethal phenotype at 30°, which is their permissive temperature. However, at their restrictive temperature (15°), a subset of the mutant α-tubulins do not support growth without wild-type *RBL2* (Table 2). Interestingly, the particular mutants that fail to suppress (*tub1-724*, *-728*, *-738*, and *-759*) have two other properties in common. First, all are of class I and arrest with no microtubules. Second, each of these specific α-tubulin mutations is synthetically lethal with Δ*rbl2* (ARCHER *et al.* 1995).

Overexpression of *PAC10* does not have an *RBL* phenotype: The requirement for either Pac10p or Rbl2p for vegetative growth could be explained if these two proteins independently carried out similar functions. To address this question, we tested if overexpressed Pac10p, like excess Rbl2p, could rescue cells from the lethality associated with excess β-tubulin. JAY47 diploid cells carry a third copy of the β-tubulin gene under the control of the inducible *GAL* promoter and integrated

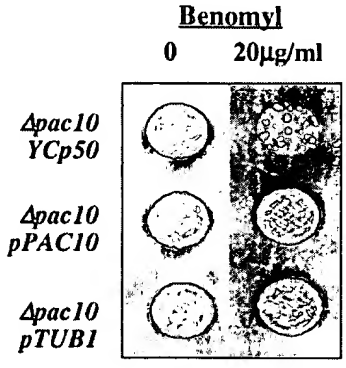

FIGURE 2.—The benomyl supersensitivity of Δ*pac10* cells is suppressed by overexpression of α-tubulin. Δ*pac10* cells (top row) fail to grow on solid medium containing 20 μg/ml benomyl. The presence of either *PAC10* (middle row) or *TUB1* (bottom row) on low-copy plasmids restores wild-type growth.

TABLE 2

Allele-specific suppression of the Δ*rbl2*, Δ*pac10* synthetic lethal interaction by overexpression of α-tubulin mutants at restrictive temperature

α-Tubulin gene	Suppression of Δ*pac10*, Δ*rbl2*	Viable with Δ*rbl2*[a]
TUB1	Yes	Yes
tub1-724 (1)	No	No
tub1-728 (1)	No	No
tub1-738 (1)	No	No
tub1-759 (1)	No	No
tub1-714 (2)	Yes	Yes
tub1-727 (3)	Yes	Yes
tub1-730 (2)	Yes	Yes
tub1-746 (3)	Yes	Yes

Double mutant cells (Δ*rbl2*, Δ*pac10*) were transformed with a series of *LEU2* plasmids bearing wild-type and mutated versions of the α-tubulin gene. The α-tubulin genes are listed by allele number, followed by their original classification in parentheses (SCHATZ *et al.* 1988; see text). The ability of each allele to suppress the synthetic lethal interaction was tested by growing at 15° on 5-FOA plates (see Figure 1). The interaction of those mutants with Δ*rbl2* previously described by ARCHER *et al.* (1995) is shown in the third column.

[a] ARCHER *et al.* (1995).

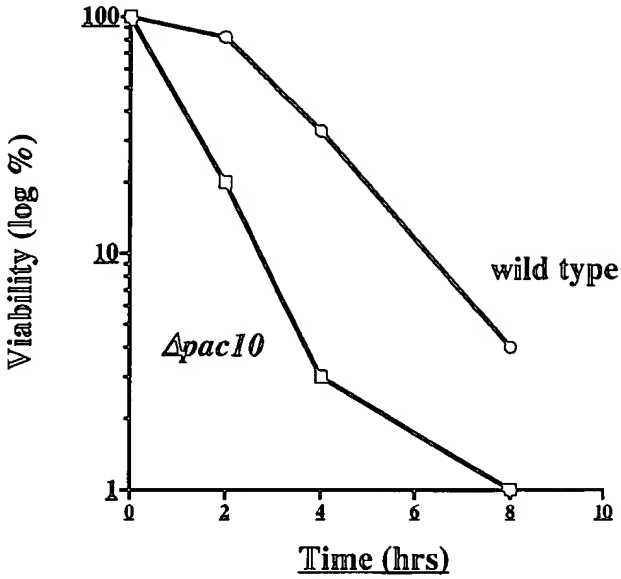

FIGURE 3.—Δ*pac10* cells are more supersensitive to overexpression of β-tubulin. Haploid cells, either "wild type" (PAY232) or "Δ*pac10*" (PAY231) and containing an extra copy of the β-tubulin gene under the inducible *GAL* promoter were grown overnight in selective raffinose media at 30°. At time 0 hr, galactose (final concentration, 2%) was added. At various times, aliquots of both cultures were counted for cell number, and appropriate fractions plated on glucose-containing medium. "Viability" represents the fraction of cells counted that gave rise to colonies.

at the normal *TUB2* locus. These cells die rapidly in medium containing galactose (ARCHER *et al.* 1995). High levels of either α-tubulin or Rbl2p rescue these cells nearly completely, and even a single extra copy of either gene provides significant rescue (10^2-fold relative to unsuppressed strains). However, *PAC10* under control of its own promoter or of the galactose promoter has no detectable effect on β-tubulin lethality.

The ability of Rbl2p to bind β-tubulin is likely to reflect some aspect of its function *in vivo*. However, we are unable to detect any physical interaction between Pac10p and β-tubulin or α-tubulin, even when both proteins are overexpressed. We searched for such complexes in extracts from cells expressing either the His_6- or HA-tagged versions of Pac10p. We analyzed those extracts using Ni-NTA beads to bind the His_6-Pac10p, or by immunoprecipitation with antibodies against the HA epitope, α-tubulin or β-tubulin. In each case, we failed to find specific association between either tubulin polypeptide and Pac10p. Both of the modified versions of Pac10p complement the *pac10* null phenotype, and therefore are functional. Under similar conditions, we can isolate Rbl2p-β-tubulin complexes (ARCHER *et al.* 1998) as well as the α-/β-tubulin heterodimer. These results suggest that Pac10p does not form a stable complex with β-tubulin.

Levels of Pac10p and sensitivity to β-tubulin: Several genetic and physiological experiments show that cells are sensitive to perturbations in the balance between α- and β-tubulins. Parallel analyses suggest that Pac10p levels affect that balance. First, overexpression of β-tubulin kills Δ*pac10* cells much more rapidly than wild-type cells (Figure 3). Four hours after induction of β-tubulin overexpression, viability of Δ*pac10* cells is 10-fold lower than that of wild-type cells. This supersensitivity to excess β-tubulin is comparable to that conferred by deletion of *RBL2* (ARCHER *et al.* 1995).

Second, increased levels of Pac10p enhance the ability of both α-tubulin and Rbl2p to rescue cells from β-tubulin overexpression (Figure 4). Typically, cells containing *GAL-TUB2* form colonies on galactose with 0.01% of the efficiency of cells plated on glucose. The presence of an extra copy of either *TUB1* or *RBL2* under control of their own promoters increases that ratio to about 2%, whereas overexpression of *PAC10* itself has no effect on survival on galactose (Figure 4). However, concomitant overexpression of *PAC10* enhances the ability of an extra copy of either *RBL2* or *TUB1* to promote growth in the presence of excess β-tubulin. When *PAC10* is present on a low-copy plasmid and under control of its own promoter, the percentage of viable colonies on galactose increases by about twofold when co-overexpressed with *TUB1* or *RBL2*. Co-overexpression of even higher levels of Pac10p, achieved using the galactose-inducible promoter, increases the viability by about eightfold. These results suggest that, although

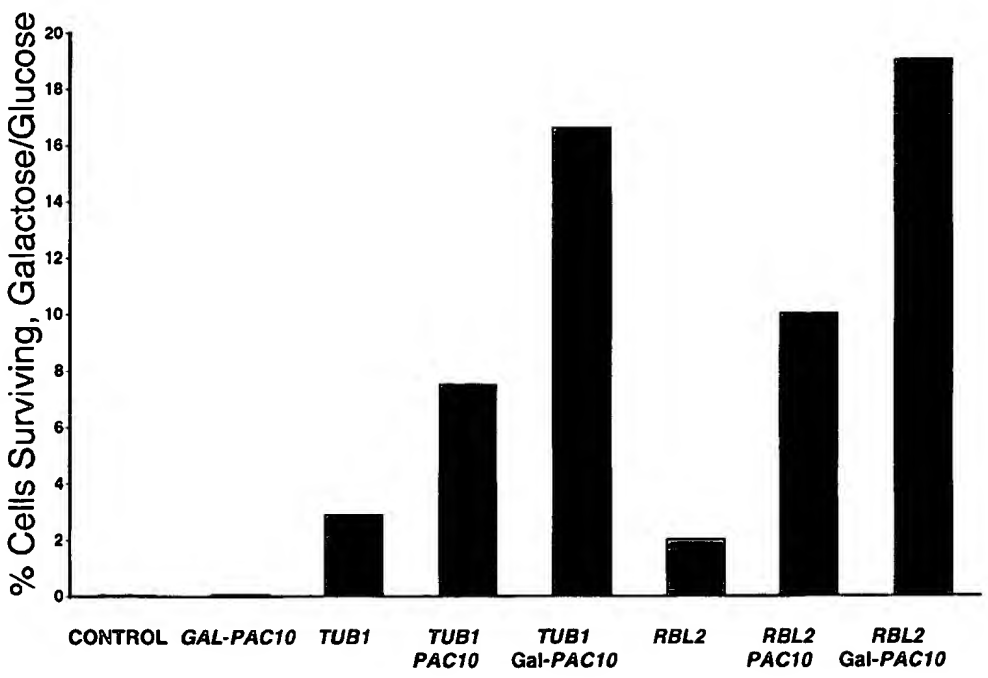

FIGURE 4.—Pac10p enhances suppression of β-tubulin lethality by Rbl2p and Tub1p. JAY47 cells (diploid cells containing a third intergrated copy of *TUB2* under control of the *GAL* promoter) were transformed with several combinations of the plasmids encoding *TUB1* (pRB539), *RBL2* (pA21A), *PAC10* (pPA36), and *GAL-PAC10* (pPA23) (Table 1). Aliquots were withdrawn from exponential phase cultures in glucose media, and the extent of suppression was calculated as a percentage of cells on galactose (inducing) *vs.* glucose (noninducing) plates that could form colonies. CONTROL is JAY47 transformed with YCpGAL. The control, and JAY47 cells expressing *GAL-PAC10*, gave ~0.01% colonies on galactose *vs.* glucose.

Pac10p cannot itself suppress β-tubulin lethality (Figure 4), it can enhance the ability of other genes to do so.

***PAC10* influence on tubulin levels:** Many of the consequences of altered Pac10p levels described above are consistent with the idea that Pac10p affects the activity of the tubulin chains *in vivo*. Accordingly, we measured α- and β-tubulin in Δ*pac10* and wild-type cells using immunoblots. The results of a typical experiment are shown in Table 3. We find that the levels of α- and β-tubulin are reduced in Δ*pac10* cells compared to wild type. However, the decrease in α-tubulin is greater, so the resulting ratio of α-tubulin to β-tubulin is ~30% lower in the mutants. The ratio is restored to its wild-type value when the mutant is transformed with a low-copy plasmid carrying the *PAC10* gene. This relationship between the ratio of tubulin chains and the presence of *PAC10* was found in four independent experiments.

Genetic interactions of *PAC10*: The data described in Table 3 provide a rationale for the synthetic lethality between Δ*pac10* and Δ*rbl2*. The absence of the β-tubulin binding activity of Rbl2p would be expected to enhance the cells' sensitivity to the imbalance in α-tubulin to β-tubulin produced by the absence of Pac10p. These relationships also rationalize the suppression of the Δ*pac10*, Δ*rbl2* synthetic lethality by *TUB1* (Figure 1), because an extra copy of that gene should provide more α-tubulin.

A direct test of this model is to analyze the effects of Δ*pac10* in other genetic backgrounds expected to alter the ratio of α- to β-tubulin. We showed previously that Δ*tub3* strains are viable, but supersensitive to benomyl (KATZ *et al.* 1990). Tub3p contributes ~15% of the cells' α-tubulin (SCHATZ *et al.* 1986b), so the properties of the Δ*tub3* strain are explicable in terms of excess β-tubulin. We transformed the Δ*tub3* strain PAY290 with the *PAC10::HIS3* fragment of pPA10 and selected for strains that had stably integrated the *HIS3* marker. The majority of those isolates were unable to lose the pPA46 plasmid (p*TUB3-CEN-URA3*). We confirmed that in those strains the chromosomal copy of *PAC10* had been disrupted using PCR Southerns. In the His^+ isolates that could lose the plasmid, the disruption fragment integrated elsewhere in the genome. These results demonstrate that the double mutant Δ*pac10*, Δ*tub3* is not viable, probably because of the presence of excess β-tubulin in these cells.

TABLE 3

Absence of Pac10p decreases the α-tubulin/β-tubulin ratio in the cell

Strain	α-Tubulin	β-Tubulin	Ratio, α/β
PAC10	3.7	3.8	0.97
Δpac10, YCp50	2.1	3.3	0.63
Δpac10, pPAC10	3.1	3.2	0.93

Cultures of wild-type cells and Δ*pac10* haploids containing the indicated plasmids were grown on glucose. Protein extracts, normalized to cell number, were analyzed for α-tubulin and β-tubulin levels by immunoblotting (see MATERIALS AND METHODS). Values were normalized to a nontubulin band in the α-tubulin immunoblot.

We also have determined whether other genes thought to participate in tubulin polypeptide metabolism interact with *PAC10*. In particular, vertebrate homologs of Cin1p and Pac2p are essential for the chaperone-mediated incorporation of denatured β-tubulin into α-/β-tubulin heterodimers *in vitro* (TIAN *et al.* 1996, 1997). Disruptions of both of these genes are lethal in strains lacking the Cin8p mitotic motor (GEISER *et al.* 1997), as is Δ*pac10*. Cin1p also has been implicated in β-tubulin folding *in vivo* (HOYT *et al.* 1997). We created diploid strains by crossing Δ*pac10* haploids (PAY170) with strains bearing deletions of either *cin1* (JFY209) or *pac2* (PAY175). The resulting diploids also contained pPA36 (p*PAC10-CEN-URA3*). Spores containing the double deletions are viable as long as the plasmid is maintained, but they are unable to grow on medium containing 5-FOA. This requirement for the *PAC10* plasmid demonstrates that both Δ*cin1* and Δ*pac2* are synthetically lethal with Δ*pac10*. A low-copy plasmid containing the *TUB1* gene rescues Δ*pac10*, Δ*pac2* and Δ*pac10*, Δ*cin1* cells (data not shown), suggesting that these synthetic lethal interactions depend at least in part on the consequences of excess β-tubulin. The results suggest that these gene products all impinge upon the same essential function, and that their proper stoichiometry is important for cell growth.

DISCUSSION

PAC10 originally was identified as a gene required for viability in the absence of the mitotic motor protein Cin8p (GEISER *et al.* 1997). However, like some of the other genes so identified, Pac10p does not appear to have motor functions, so its absence could act indirectly to exacerbate the sublethal consequences of a *CIN8* deletion. Our independent identification of *PAC10* as important for cellular functions involving the tubulin chains supports this view. The results presented above also give some insight into those functions and how *PAC10* may participate in them. Specifically, we find that cellular α-tubulin levels, and consequently the α-/β-tubulin ratio, are affected by levels of Pac10p. Previous studies demonstrate that a depressed ratio of α-/β-tubulin affects microtubule function adversely. We hypothesize that this defect in combination with either the absence of the Cin8p motor protein or of the Rbl2p β-tubulin binding protein may severely disrupt essential microtubule functions.

Levels of Pac10p are important for *in vivo* microtubule functions: In the absence of Pac10p, cells become supersensitive to the microtubule depolymerizing drug benomyl, also a property of cells that have a small deficit in α-tubulin (KATZ *et al.* 1990). Similarly, *pac10* nulls are dependent upon the presence of the β-tubulin-binding protein Rbl2p for growth. Both of those phenotypes are substantially suppressed by excess α-tubulin. Those data are explicable if a consequence of the absence of Pac10p is a decrease in α-tubulin levels, and thus a reduced capacity to bind β-tubulin and so suppress its toxic effects. That deletion of *PAC10*, like deletion of *RBL2*, renders cells supersensitive to β-tubulin overexpression supports that interpretation.

However, Pac10p does not form a stable complex with β-tubulin. We cannot detect a physical association between Pac10p and either tubulin chain under conditions where we can readily isolate both Rbl2p-β-tubulin complexes and the α-β tubulin heterodimer itself. An *in vivo* test of β-tubulin binding also fails for Pac10p: even *GAL*-induced expression of *PAC10* does not increase the ability of cells to survive induced overexpression of β-tubulin under conditions in which both α-tubulin and Rbl2p act as strong suppressors. Therefore, the effect of deletion of *PAC10* on sensitivity to β-tubulin is likely exercised indirectly.

Deletion of *PAC10* changes the stoichiometry of the tubulin chains. The levels of both tubulin chains are decreased in the mutant cells, but the decline in α-tubulin is greater, so that the α/β ratio decreases by 30%. The balance of tubulin polypeptides is tightly regulated at the level of protein. For example, cells carrying a single extra copy of *TUB1* do display a proportional increase in the amount of α-tubulin mRNA, but the α-tubulin polypeptide level is very nearly the same as in wild-type cells (KATZ *et al.* 1990). Presumably, the α-tubulin synthesized in these cells that is in excess of the β-tubulin complement is unstable and degraded. Perhaps in the case of Δ*pac10* cells, then, the decreased levels of α-tubulin result in undimerized β-tubulin. The diminished α/β tubulin ratio could explain the several phenotypes of *pac10* nulls and the ability of α-tubulin overexpression to suppress those phenotypes.

β-tubulin lethality and its suppression: Excess β-tubulin is much more toxic than either excess α-tubulin or excess heterodimer (BURKE *et al.* 1989; WEINSTEIN and SOLOMON 1990). Presumably, undimerized β-tubulin, but not undimerized α-tubulin, competes with the heterodimer for binding to factors essential for microtubule assembly and cell growth (WEINSTEIN and SOLOMON 1992). This model is formally analogous to the balance of components hypothesis, which illuminated the consequences of altered stoichiometries of components in phage morphogenesis (FLOOR 1970; STERNBERG 1976). We do not yet know the identity of the targets of free β-tubulin.

The suppression of β-tubulin lethality by α-tubulin or Rbl2p is likely to be based on their ability to bind the free β-tubulin. Although the details of these interactions are not understood, we do know that the suppression has at least one striking feature. Cells containing β-tubulin, and a low-copy plasmid bearing either *RBL2* or *TUB1* under control of their own promoters, show about 1% suppression of β-tubulin lethality, 100-fold greater than the 0.01% of the cells without the plasmid. Surprisingly, those suppressed cells form colonies the same size as those formed by wild-type cells. Clearly, then, nearly all of the products of each mitosis must be viable. If the

proportion of viable mitotic products was lower—for example, 1%—the colonies would be much smaller. This behavior may mean that once cells pass over a threshold event, they can survive excess β-tubulin. According to this model, the presence of the suppressor increases the probability that they will pass over such a threshold.

This model also provides us with a way of thinking about the effects of increased *PAC10* expression on survival of excess β-tubulin. We note that overexpression of *PAC10* enhances the suppression of β-tubulin lethality by modest increases in the levels of Rbl2p and α-tubulin, increasing survival by two- to eightfold. Although overexpression of *PAC10* is itself not sufficient to increase survival, it may provide sufficient α-tubulin to act cooperatively with excess Rbl2p or α-tubulin.

The molecular role of *PAC10*: Pac10p and Rbl2p do not appear to have redundant functions. We do not yet know in what way Pac10p acts to affect levels of α-tubulin protein. It shows no structural relationship to transcription factors, and thus is unlikely to affect α-tubulin mRNA synthesis. As noted by its original identifiers, Pac10p does have homologs in other organisms, including humans where it is believed to bind to a tumor suppressor gene product that itself has no obvious homolog in *S. cerevisiae* (GEISER *et al.* 1997). It may interact with α-tubulin mRNA or protein to stabilize them, although we have been unable to identify a stable complex with the latter. It may also be involved in folding of α-tubulin, although no homolog among the proteins essential in the *in vitro* assay for such activities is known (TIAN *et al.* 1996). Experiments to distinguish among those possibilities are in progress.

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integrity. The recent discovery of linker proteins bridging between IFs and other cytoskeletal components and their importance to cell survival and genetic disease open a new field for understanding the functional interactions among the structural elements within the cytoplsm.

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Kinesin and Dynein Superfamily Proteins and the Mechanism of Organelle Transport

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Cells transport and sort proteins and lipids, after their synthesis, to various destinations at appropriate velocities in membranous organelles and protein complexes. Intracellular transport is thus fundamental to cellular morphogenesis and functioning. Microtubules serve as a rail on which motor proteins, such as kinesin and dynein superfamily proteins, convey their cargoes. This review focuses on the molecular mechanism of organelle transport in cells and describes kinesin and dynein superfamily proteins.

Neurons and epithelial cells are among the many types of cells that develop polarized structures. The neuron is composed of a cell body, dendrites, and a long axon along the direction of impulse propagation. The axon lacks protein synthesis machinery, and thus all the proteins required in the axon and synaptic terminal must be transported down the axon after they are synthesized in the cell body. Most proteins are conveyed in membranous organelles or protein complexes. In this sense, organelle transport in the axon is fundamentally important for neuronal morphogenesis and functioning. Because similar mechanisms are observed in other cells, the neuron serves as a good model system to study the general mechanisms of organelle transport (*1*). Epithelial cells also develop polarized structures, that is, the apical and basolateral regions, to which certain proteins are specifically transported and sorted (*2*).

Microtubules are 25-nm tubule-like structures formed by α,β-tubulin heterodimers. Thirteen parallel protofilaments composed of linearly arranged heterodimers form the

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microtubule wall, to which various microtubule-associated proteins and motor proteins bind. The microtubule is a polar structure with a fast-growing or plus end and a minus end.

Microtubules serve as rails for the transport of organelles and are organized in a regular manner in these polarized cells. In nerve axons, the microtubules are arranged longitudinally with the plus end pointing away from the cell body, whereas in epithelial cells microtubules are organized with the plus end pointing toward the basement membrane. In most other cells such as fibroblasts and macrophages, microtubules radiate from the cell center with the plus ends pointing toward the periphery. In all of these cells, various organelles are transported along the microtubules by means of microtubule-associated motor proteins.

Early light microscopy studies of living nerve axons and biochemical studies of axonal transport revealed membranous organelles moving by fast flow (3). Electron microscopy (EM) studies suggested the presence of short cross-bridge structures between the organelles and microtubules, which are candidates for microtubule-associated motor proteins conveying the membranous organelles along microtubules (4) (Fig. 1). Video-enhanced differential interference contrast microscopy combined with biochemical analyses revealed the presence of a microtubule plus end–directed motor protein, kinesin, a microtubule-activated adenosine triphosphatase (ATPase) of 380 kD (5, 6). The kinesin molecule consists of two 120-kD kinesin heavy chains (KHCs) and two 64-kD kinesin light chains (KLCs) (6). It has a rod-like structure composed of two globular heads (10 nm in diameter), a stalk, and a fan-like end, with a total length of 80 nm. The globular heads are composed of KHCs that bind to microtubules (7, 8) (Fig. 2); the KLCs constitute the fan-like end (7). Complementary DNA (cDNA) encoding *Drosophila* KHC yields a protein of 975 amino acids in which the NH_2-terminal ~350 amino acids form the motor domain (which binds to microtubules), an α-helical coiled coil–rich stalk domain involved in dimer formation, and a tail domain (9). Localization and functional assays indicate that kinesin acts as a plus end–directed microtubule

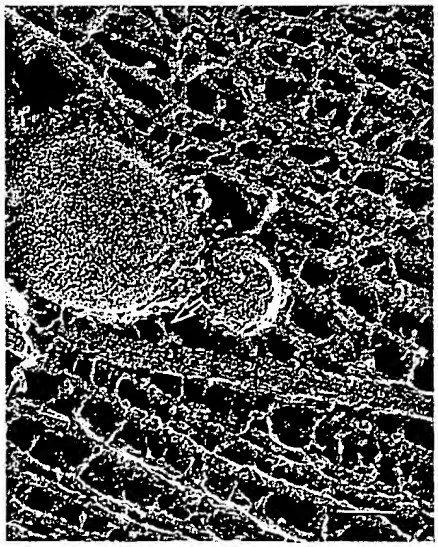


Fig. 1. Quick freeze–deep etch electron micrograph of mouse axon. A membranous organelle conveyed by fast transport is linked with a microtubule by a short cross-bridge (arrow), which could be a motor molecule. Scale bar, 50 nm.

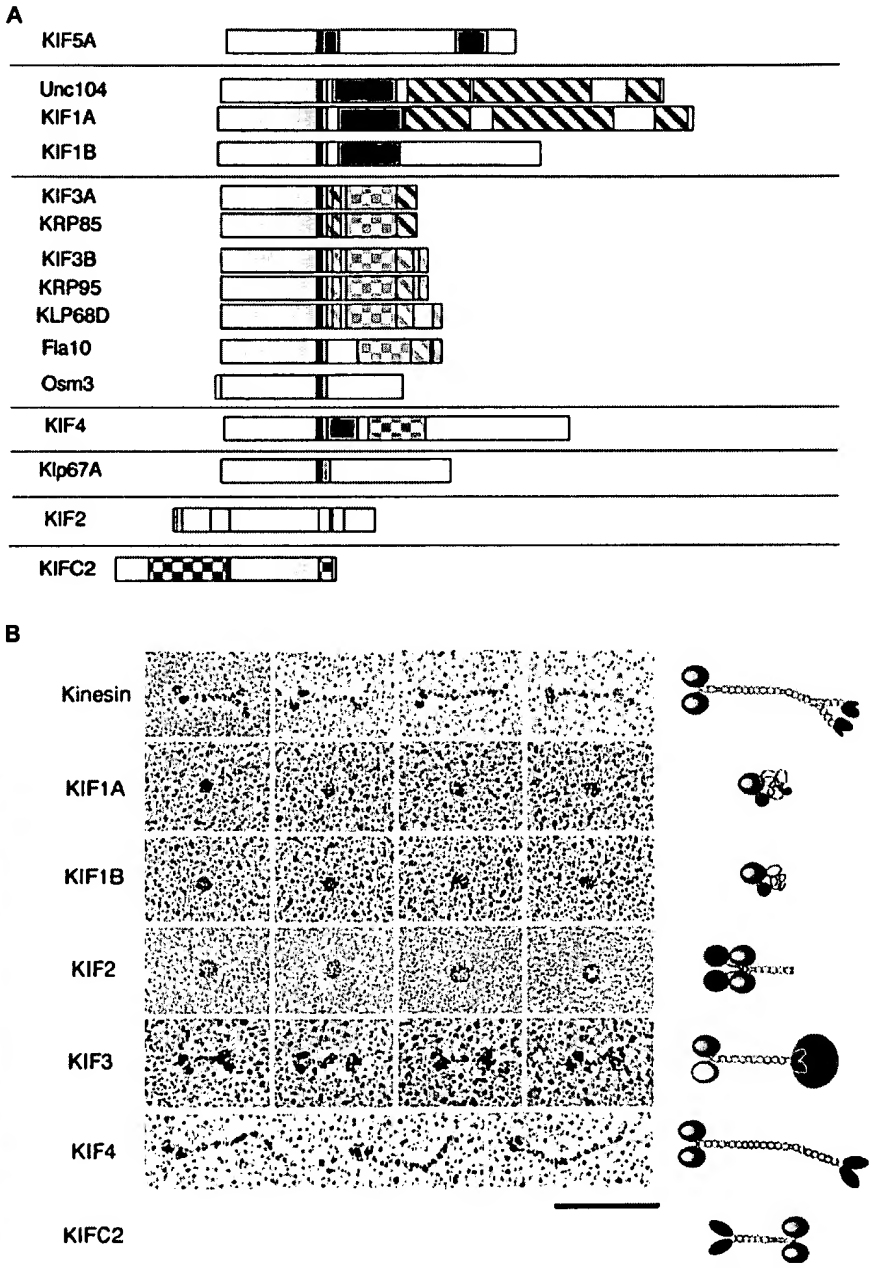


Fig. 2. (**A**) Schematic representation of kinesin superfamily proteins described in the text. Conserved motor domains are aligned and colored pink. Dark blue and red regions correspond to the N-type and C-type (KIFC consensus) specific neck regions, respectively. Yellow rectangles indicate the M-type specific neck and tail regions. Other conserved regions within each class, family, or subfamily are indicated by solid rectangles, oblique stripes, or checks, respectively, with different colors. (**B**) Left: Panels of main members of KIFs functioning in organelle transport, as observed by low-angle rotary shadowing EM. Scale bar, 100 nm. Right: Schematic illustrations of the same KIFs, based on EM studies or predicted from analysis of primary structures.

motor involved in anterograde membrane transport (*10–12*).

Another microtubule-activated ATPase that promotes transport in the opposite direction, known as brain dynein or cytoplasmic dynein, was discovered in 1987. Cytoplasmic dynein is composed of two heavy chains of 530 kD each, three intermediate chains of 74 kD each, and four light intermediate chains of 55 to 60 kD each, and moves along a microtubule from the plus end to the minus end, making it a good candidate for a motor for retrograde axonal transport (*13*). In living axons, however, various types of membranous organelles (including synaptic vesicle precursors and vesicles containing synaptic and axonal plasma membrane precursors) are transported anterogradely, whereas multivesicular bodies and endosomes are transported retrogradely, with both types of transport occurring at distinct velocities. Mitochondria are transported in both directions (*1*, *3*).

The kinesin superfamily of proteins plays a major role in this complex organelle transport. A systematic molecular biological search of kinesin superfamily genes coding for proteins containing adenosine triphosphate (ATP)–binding and microtubule-binding consensus sequences led to the discovery of new kinesin superfamily proteins related to organelle transport (KIFs), 11 from mouse brain (*1*, *14*) and three from *Drosophila* (*15*). Motor proteins from *Caenorhabditis elegans* were identified in mutants with slow and uncoordinated movement [for example, Unc104 (*16*)] or chemotaxis [Osm3 (*17*)]. Further motor proteins ($KRP_{85/95}$) have been identified in biochemical extracts from sea urchin (*18*). Systematic molecular biological searches have identified at least two or three members of the dynein superfamily proteins related to the transport of organelles in sea urchin (*19*), rat (*20*), and human (*21*).

In this review, I describe well-characterized kinesin and dynein superfamily proteins and their function in organelle transport in cells. Some other members of these superfamilies are also involved in mitosis or meiosis. [See (*22*, *23*) for comprehensive recent reviews of the motor proteins involved in cell division and the mechanism of motility of motor proteins.]

The Kinesin Superfamily Proteins

Three major types of kinesin superfamily proteins have been identified according to the position of the motor domain: NH_2-terminal motor domain type, middle motor domain type, and COOH-terminal motor domain type (referred to below as N-type, M-type, and C-type, respectively). Of the proteins that have been identified, the KHC, Unc104/KIF1, KIF3/$KRP_{85/95}$, KIF4, and Klp67A families (N-type), the KIF2 family (M-type), and the KIFC2/C3 family (C-type) are involved in organelle transport (Figs. 2 and 3).

N-type proteins: Conventional kinesins. Conventional KHC itself forms a family (Fig. 3). Although three members of this family have been identified in mouse (KIF5A, KIF5B, and KIF5C) (*1*, *14*, *24*) and two in humans (HsuKHC and HsnKHC) (*25*), only one member has been identified in other metazoans such as sea urchin, *Drosophila*, and *C. elegans* (*26*). KIF5B and HsuKHC are expressed ubiquitously in many tissues, whereas KIF5A, KIF5C, and HsnKHC are specific to nerve tissue.

Kinesin is primarily associated with anterogradely transported membranous organelles in nerve axons (*11*). In various types of cells, kinesin associates with the endoplasmic reticulum (ER), Golgi complex, mitochondria, endosomes, and lysosomes (*22*). Several distinct approaches have been adopted to elucidate the function of kinesin. The *Drosophila* KHC gene mutants do not survive beyond the larval stage and exhibit loss of mobility and tactile response. Thus, KHC in *Drosophila* transports proteins important for action potential propagation, whereas KHC mutants exhibit no apparent change in the concentration of synaptic vesicles in nerve terminal cytoplasm (*26*).

Various kinds of functional studies, including antisense studies (*27*), microinjection of blocking antibodies (*12*, *28*, *29*), and transfection of KHC cDNA containing a point mutation of the ATP-binding domain (*30*), have resulted in a variety of phenotypes in cells, sometimes conflicting with each other (*27–29*). In summary, however, it is reasonable to conclude that kinesin is responsible for the microtubule plus end–directed transport of membranous organelles, which is important for the neurite outgrowth of neurons, and for microtubule plus end–directed lysosome transport in various types of cells. Further studies are needed to characterize the cargoes of kinesin in vivo.

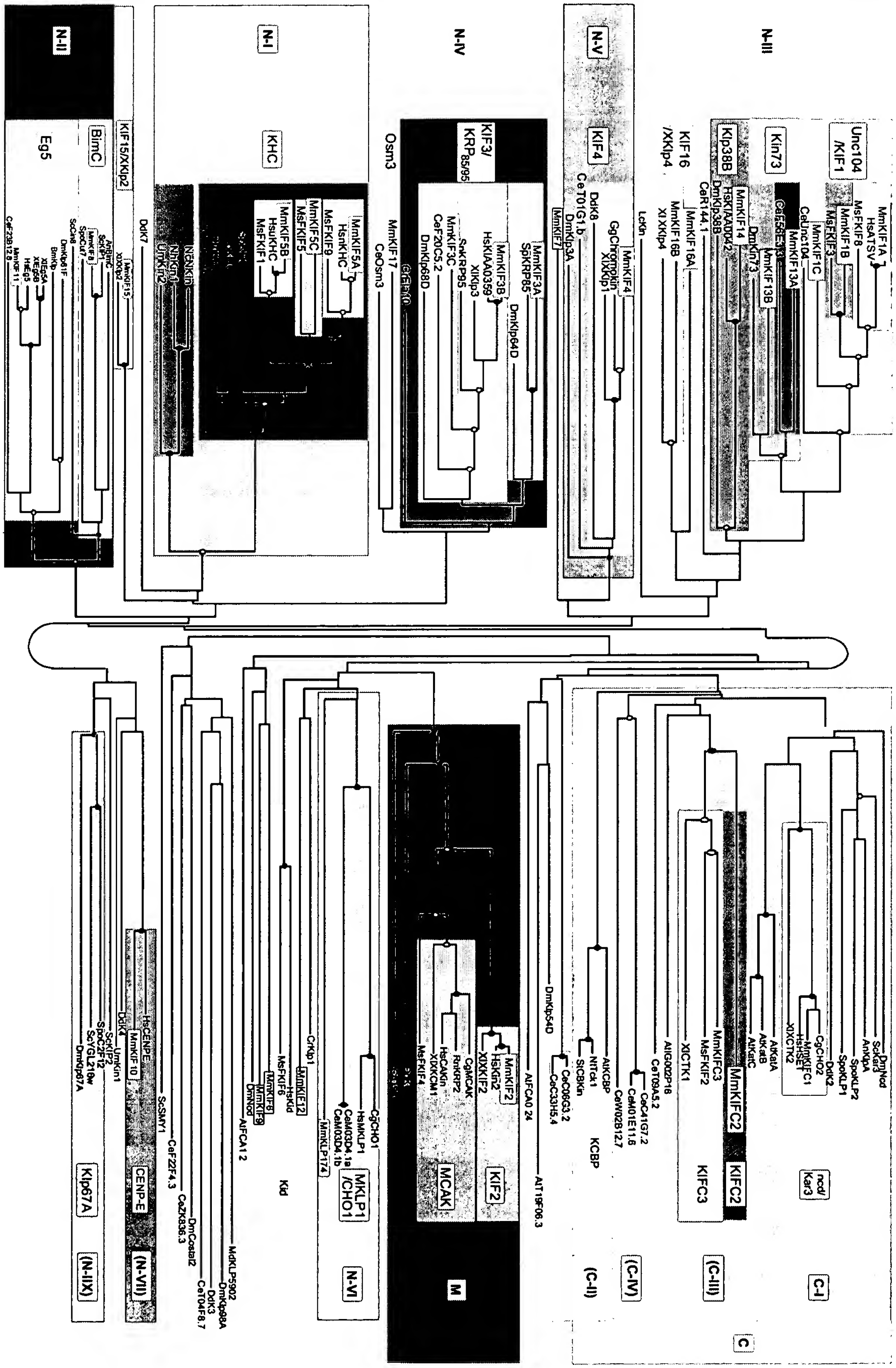
Because KLCs are localized at the fan-like end of kinesin where it binds to membranous organelles, it has been speculated that KLCs modulate the binding of cargoes to microtubules (*7*). KLC cDNAs from several organisms were cloned and sequenced (*31*). Three isoforms encoding peptides composed of 542 to 560 amino acids were identified in rat, and alternative splicing showed that they are derived from a single gene (*31*). The overall structure of KLC has been conserved among various species, and a long series of NH_2-terminal heptad repeats and several imperfect tandem repeats closer to their COOH-termini were identified in KLC. Antibodies to KLCs, especially to tandem repeats, inhibit fast axonal transport in the axoplasm and release kinesin from purified membrane vesicles in vitro; this finding supports the idea that KLCs play a role in the interaction of kinesin with membranes (*32*). On the other hand, KLCs may regulate the ATPase activity of KHCs. In EM images, kinesins have sometimes been observed folded (*7*). The ATPase activity of KHCs alone is greater than that of KHCs complexed with KLCs (*33*). When kinesin binds membranous organelles to microtubules, its head domains are apart from the tail, whereas in the absence of microtubules kinesin may be folded, bringing KHC and KLC close to each other and possibly allowing KLC to regulate the ATPase activity of KHC in vivo (*33*).

N-type proteins: Fast anterograde monomeric motors—the Unc104/KIF1 family. Mutations in the *unc104* gene of *C. elegans* result in uncoordinated and slow movement of the nematode; *unc104* cDNA encodes a 1584–amino acid N-type motor protein. Unc104 mutants exhibit few synaptic vesicles in nerve terminals and form few synapses, and the neuron cell bodies accumulate similar vesicles tethered together within the cytoplasm. Thus, Unc104 is thought to be a neuron-specific motor used for the anterograde transport of synaptic vesicles along axonal microtubules (*16*).

The mouse homolog of Unc104, KIF1A, is a globular molecule with a diameter of 14 nm and exists as a monomer (*34*) (Figs. 3 and 4). KIF1A, expressed specifically in neurons and enriched in axons, is the fastest moving microtubule plus end–directed motor protein in mammals (1.2 to 1.5 μm/s in vitro) involved in anterograde transport in axons. The cargo of KIF1A includes a subset of precursors for synaptic vesicles containing synaptophysin, synaptotagmin, and Rab3A (*34*). Interestingly, the cargo of KIF1A does not contain SV2, a synaptic vesicle protein and transmitter transporter (*34*).

Knockout mice lacking KIF1A show sensory and motor disturbances and a marked reduction in the density of synaptic terminals and synaptic vesicles in nerve terminals, and they accumulate clear vesicles in nerve cell bodies. In addition, focal neuronal cell death and secondary degeneration of axons are observed in the central nervous system of these mice, and primary cultures of $KIF1A^{-/-}$ neurons exhibit prominent neuronal cell death in vitro (*35*). Thus, KIF1A (and possibly Unc104) is a unique monomeric anterograde motor for the transport of a subset of synaptic vesicle precursors, and it plays an important role in neuronal function and neuronal survival (Figs. 2 to 4). Another member of the Unc104/KIF1A family, KIF1B, is thought to function as a monomeric motor for the anterograde transport of mitochondria (*36*) (Figs. 2 to 4).

Thus, the members of this family are


N-II
N-I
N-IV
N-V
N-III
Eg5
BimC
KIF15/XKlp2
KHC
KIF3/KRP85/95
Osm3
KIF4
KIF16/XKlp4
Klp38B
Kin73
Unc104/KIF1
Klp67A
(N-IIX)
CENP-E
(N-VII)
Kid
MKLP1/CHO1
N-VI
MCAK
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KCBP
(C-II)
(C-IV)
KIFC3
(C-III)
KIFC2
ncd/Kar3
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C

mostly monomeric microtubule plus end–directed motor proteins with a variety of distinct cargoes. How this monomeric single-headed motor moves on a microtubule is an important and intriguing question, because the hand-over-hand mechanism cannot be used, as with the dimeric, two-headed motors (*1*, *37*).

N-type proteins: Anterograde heterodimeric motors—the KIF3/KRP$_{85/95}$ family. Another group of two-headed anterograde motors includes the KIF3/KRP$_{85/95}$ family (Figs. 2 and 3). A KIF3A-KIF3B heterodimer (KIF3A/3B) assembles with KAP3 (kinesin superfamily associated protein–3), forming a heterotrimeric motor with a plus end–directed microtubule sliding activity at a velocity of ~0.3 μm/s (*14*, *38*, *39*). The motor is expressed ubiquitously and is used for the anterograde transport of membranous organelles, 90 to 160 nm in diameter, distinct from synaptic vesicle precursors and from vesicles carried by other motors such as kinesin and KIF2 (*38*) (Figs. 2 to 4).

KAP3 is a globular protein (~11 nm in diameter) that binds to the tail domain of KIF3A/3B. KAP3 binding does not affect the ATPase activity of KIF3A/3B, but it is associated with the membrane-bound form of KIF3A/3B (through the tail domain of KIF3A/3B) and is thought to regulate the membrane binding of the KIF3 heterodimer (*39*) (Fig. 2). The human homolog of KAP3 has been shown to be a small–molecular weight G protein GDP dissociation stimulation factor (SmgGDS)–associated protein that is phosphorylated by Src tyrosine kinase and regulates the interaction of a group of small G proteins with membranes (*40*). The *Drosophila* homolog of KIF3B, Klp68D, is also thought to play a role in anterograde axonal transport (*41*). The sea urchin homolog of KIF3A/3B, KRP$_{85/95}$, in association with KAP115 (also named kinesin II) (*42*), is thought to deliver ciliary components, most likely protein complexes that are required for the elongation of cilia and for the formation of a stable central pair of cilial microtubules (*43*) (Fig. 3).

In *Chlamydomonas*, analysis of *fla10* mutants defective in flagellar assembly led to the identification of the *fla10* gene encoding another member of the KIF3/KRP$_{85/95}$ family, KHP1 (Fla10) (*44*) (Figs. 2 and 3). KHP1 is required for the movement of particles within flagella and for the transport of inner dynein arms, subunit p28^{IDA4}, to the tip of flagella (*44*, *45*).

A final member closely related to the KIF3/KRP$_{85/95}$ family is exemplified by the *C. elegans osm-3* mutant, which has defects in chemosensory responses such as osmotic stress avoidance and chemotaxis (*17*). The dendritic sensory cilia of certain groups of sensory neurons in amphid and phasmid sensilla are foreshortened. Osm3 probably transports materials necessary for the growth of sensory cilia in sensory neurons (*17*) (Fig. 3). In summary, motors of this family (KIF3A/3B, KRP$_{85/95}$, and probably Klp68D/64D) form a heterotrimer with an associated protein KAP.

N-type proteins: KIF4 and Klp67A families. Another family of motors is illustrated by KIF4 (*14*, *46*). KIF4 messenger RNA (mRNA) is expressed abundantly in juvenile tissues, including differentiated young neurons; in adult mice, its expression is considerably decreased, except in the spleen. KIF4 is colocalized with membranous organelles in the growth cones of differentiated neurons as well as in the cytoplasm of cultured fibroblasts. During the mitotic phase of the cell cycle, KIF4 appears to colocalize with membranous organelles in the mitotic spindle. Hence, KIF4 is a microtubule plus end–directed anterograde motor for the transport of a certain group of membranous organelles in juvenile neurons and other cells (*46*) (Figs. 2 to 4). Chromokinesin, the chicken isolog of KIF4, contains a basic leucine zipper DNA binding domain, is associated with chromosome arms, and functions as a mitotic motor with DNA as its cargo (*47*) (Fig. 3). Because KIF4 also contains a DNA binding domain similar to that of chromokinesin, KIF4 may have an additional function such as the transport of mRNA in certain kinds and developmental stages of cells.

A final N-type motor is *Drosophila* Klp67A. It is a microtubule plus end–directed motor (0.05 μm/s) (Figs. 2 and 3) that acts as a mitotic motor and may play a role in positioning mitochondria near the mitotic spindle pole (*48*).

M-type proteins: The KIF2 family. KIF2 (716 amino acids, relative molecular mass M_r = 80,945) is a unique M-type KIF (*14*, *49*). The NH_2-terminal domain of 189 amino acids is predicted to be globular and the central motor domain is also predicted to be largely globular, whereas the COOH-terminal region is predicted to be largely α-helical. KIF2 forms a homodimer, a globular molecule with a diameter of ~16 nm (Figs. 2 and 3). KIF2 is a microtubule plus end–directed motor (~0.4 μm/s) and is expressed ubiquitously. It is abundantly expressed in developing axons, whereas the extent of expression decreases in adults. The cargo of KIF2 is vesicles about 100 to 120 nm in diameter, enriched in the neuronal growth cone and distinct from the membranous organelles carried by other motors (*49*). The cargo of KIF2 includes the specific form of β subunit of the insulin-like growth factor–1 (IGF-1) receptor (βgc), and the treatment of PC12 cells with KIF2 antisense RNA inhibits neurite outgrowth in PC12 cells (*50*). Thus, KIF2 is a unique M-type KIF anterogradely transporting vesicles important for axonal extension in developing neurons (Figs. 2 to 4).

C-type proteins: The KIFC2/C3 family. Several C-type motors, such as ncd in *Drosophila* and Kar3 in *S. cervisiae*, are motors for meiosis, mitosis, and karyogamy (*51*) (Fig. 3). These family members show microtubule minus end–directed motility. Because the only microtubule minus end–directed motor identified for organelle transports is cytoplas-

Fig. 3. (facing page) Phylogenetic tree of kinesin superfamily proteins. Superclass, class, family, and subfamily designations are based on molecular phylogenetic analysis of information derived from the motor domain and multiple sequence comparisons of the nonmotor domain of each kinesin superfamily protein, as follows: The superclass was defined by the existence of the conserved neck consensus (~10 amino acids) adjacent to the motor domain. For many KIFs, this superclass corresponds to the position of the motor domain in the molecule; superclasses N, M, and C correspond to N-type, M-type, and C-type KIFs. Class was defined by the class-specific consensus domains or regions that are often found adjacent to the superclass consensus neck. Eight classes (N-I, N-II, N-III, N-IV, N-V, N-VI, N-VII, and N-IIX) in superclass N, one class (M) in superclass M, and four classes (C-I, C-II, C-III, and C-IV) in superclass C were identified. Class numbers and family and subfamily names are derived from the time of discovery and characterization of the genes. Sequences of KIFs registered in the public databases (GenBank/EMBL/DDBJ DNA database or PIR/SwissProt/PRF protein database) by 27 August 1997 were analyzed by Clustal alignment and the neighbor-joining method. (Details of the phylogenetic analyses are available on our World Wide Web page, http://cb.m.u-tokyo.ac.jp/KIF.) The name of each KIF consists of the acronym of the species name followed by its conventional name or registered name in the database. For the sequences identified in the genome project, the cosmid name is used. For example, mouse (*Mus musculus*) protein KIF1A is designated as MmKIF1A, and the *C. elegans* sequence found as the third protein coded in cosmid F56E3 is designated as CeF56E3.3. Thus, it is easy to access database entries using these names and the program DBGET; accession numbers are also available on our Web page. Nodes with >950 of 1000 bootstrap values are marked with solid red circles; nodes with >900 of 1000 bootstrap values are marked with open red circles. The clusters identified with this analysis are color-coded. For example, the large light blue rectangle at the upper left marks class N-III, which contains Unc104/KIF1 (pink), Kin73 (orange), Klp38B (blue), and KIF16/XKlp4 (salmon) families; in turn, the Unc104/KIF1 family consists of three subfamilies: KIF1A (yellow), KIF1B (blue), and KIF1C (green). Names of families and their members referred to in this review and related to organelle transport are in larger type. For the family name, the name of the founding member (the first cloned and characterized member) is adopted.

mic dynein, it is reasonable to assume that as yet unidentified C-type KIFs exist for retrograde transport. On the basis of the discovery of a COOH-terminal KIF consensus sequence at the neck region upstream of the head region (*52*) and the results of a polymerase chain reaction using a motor domain gene consensus sequence (*52*, *53*), three C-type KIFs have been identified in mouse brain (*24*, *52–54*). KIFC2 forms a homodimer without associated polypeptides. It exhibits a microtubule-activated ATPase activity (*51*, *52*) and is localized mainly in the cell body and dendrites (*52*). The cargo of KIFC2 was identified as a new kind of multivesicular body–like membranous organelle, distinct from conventional multivesicular bodies, that functions as a shuttle between early and late endosomes (*52*). Because KIFC2 is mainly localized in the cell body and dendrites, even after the overexpression of KIFC2 in primary cultured neurons, it is thought to be a unique C-type motor that mainly functions in the dendritic transport of multivesicular body–like membranous organelles (Figs. 2 and 4). The amino acid sequence analysis of KIFC2 strongly suggests that it moves along microtubules toward the minus end, but this cannot be firmly established without an in vitro motility assay (*52*, *53*).

New KIFs. Further KIFs are likely to play roles in organelle transport, such as KIFs conveying synaptic plasma membrane proteins essential for vesicle docking (for example, SNAP25 and syntaxin 1A), KIFCs as retrograde motors in axons, and slow transport motors carrying cytoskeletal proteins and cytosolic proteins. A systematic search for cDNAs encoding as yet unidentified KIFs has been performed, revealing a total of 18 new KIFs (KIF3C, 6, 7, 8, 9, 10, 11, 12, 13A, 13B, 14, 15, 16A, 16B, 17, 22, C3, and C4) (*24*, *54*) (Fig. 3). On the basis of the expression of their mRNA in tissue, these KIFs could play important roles in organelle transport in many types of cells, including neurons and epithelial cells.

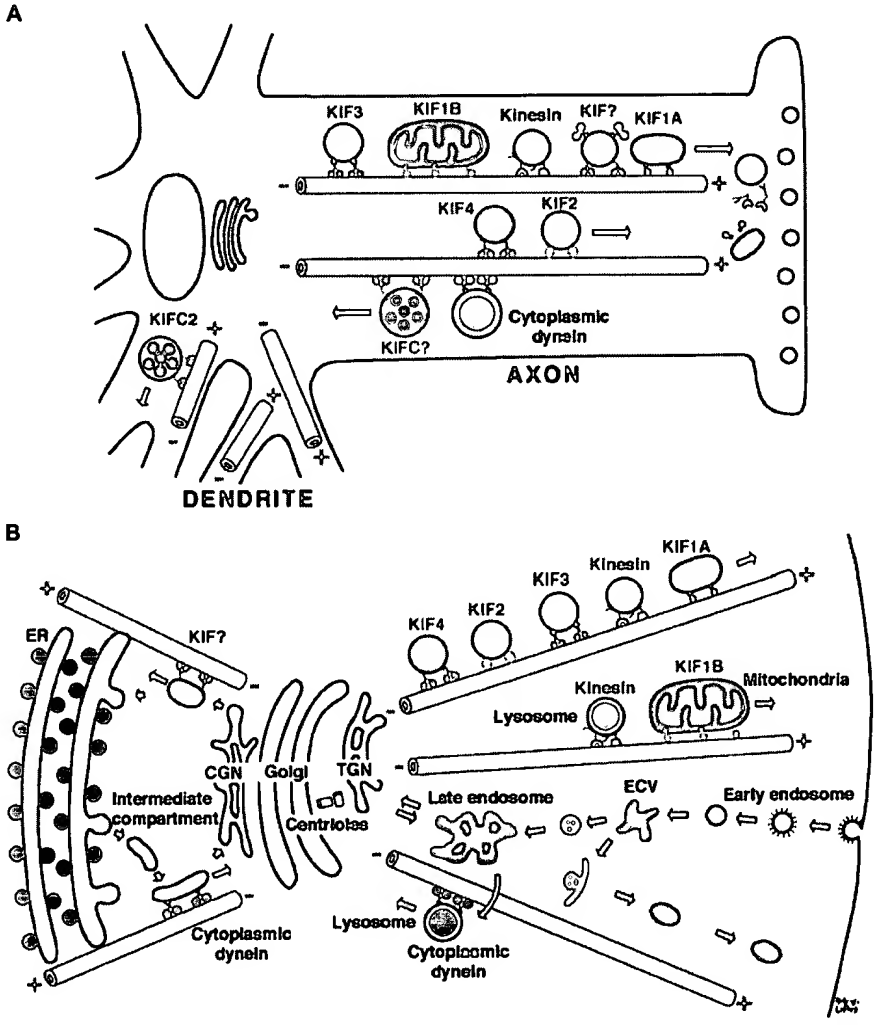

Fig. 4. Scheme of KIFs and cytoplasmic dynein and their cargo organelles in nerve axons (**A**) and in cells in general (**B**). In neurons, KIF2 and KIF4 work mostly in juvenile stages. In (B), neuron-specific KIFs and ubiquitous KIFs are drawn in the same cell. CGN, cis-Golgi network; TGN, trans-Golgi network; ECV, endosomal carrier vesicle. Black arrows indicate the direction of transport.

The Dynein Superfamily Proteins

Cytoplasmic dynein is a member of the dynein superfamily of proteins. It is a massive multisubunit complex (1.2 MD) composed of two heavy chains (~530 kD), three intermediate chains (74 kD), and four light intermediate chains (~55 kD), and moves along microtubules toward their minus ends (*13*, *23*) (Fig. 5). The cytoplasmic dynein heavy chain consists of 4644 amino acids in rat and 4092 amino acids in *Dictyostelium* and yeast, and it contains phosphate-binding pockets (P-loops) in its central region (*23*, *55*). On the basis of a comparison of the primary structure of cytoplasmic dynein with that of axonemal dynein, the central and COOH-terminal regions are predicted to form a globular domain interacting with microtubules and having motor activity, and the NH_2-terminal region is thought to be the site of the binding of cargoes (*55*) (Figs. 4 and 5).

In addition to intermediate and light intermediate chains, cytoplasmic dynein is associated with the protein complex dynactin (*56*). Dynactin contains 10 subunits: $p150^{Glued}$, $p135^{Glued}$ (a brain-specific variant of $p150^{Glued}$), p62, dynamitin (p50), actin-related protein 1 (Arp1), actin, actin-capping protein α subunit, actin-capping protein β subunit, p27, and p24, with a stoichiometry of 1:1:1:4:9:1:1:1:1:1. The $p150^{Glued}$-$p135^{Glued}$ heterodimer forms a side-arm projection from an Arp1-actin short filament (37 nm in length) and two small globular heads containing a microtubule binding site (*57*). The NH_2-terminal region of $p150^{Glued}$ forms a side arm that interacts with the 74-kD cytoplasmic dynein intermediate chain (*58*). Dynamitin probably links $p150^{Glued}$ to the Arp1-actin short filament, which possibly links cytoplasmic dynein to its cargo through $p150^{Glued}$. Thus, cytoplasmic dynein, especially its 74-kD intermediate chain, is probably linked to its cargo through the $p150^{Glued}$–Arp1-actin short filament complex, and the binding of $p150^{Glued}$ to Arp1 is mediated by dynamitin (*23*, *57*, *58*) (Fig. 5). Interestingly, $p150^{Glued}$ alone can bind to microtubules.

How does cytoplasmic dynein function in organelle transport? Immunocytochemical analysis, in vitro motility assays, antibody injection, and subcellular fractionation suggest that cytoplasmic dynein is a motor for the retrograde transport of membranous organelles in axons (*13*, *59*), the

distribution of late endosomes and lysosomes (60), the centrosomal localization of the Golgi complex (61), the vesicular transport from early to late endosomes (62), the apical transport of Golgi-derived membranes in intestinal epithelial cells (63), and the movement of phagosomes (64). Inhibition of the activity of dynactin by overexpressing dynamitin—which probably leads to the dissociation of cytoplasmic dynein from its cargoes—resulted in the dispersion of the Golgi complex, the redistribution of early and late endosomes toward the cell periphery, and suppression of the transport of intermediate compartment from the ER to the Golgi (65, 66) (Fig. 4).

All these functions could be related to members of the cytoplasmic dynein family. At present, several multiple cytoplasmic dynein heavy chains have been identified—at least two (CyDn and DLP4) in rat (20), two (DHC1A and DHC1B) in sea urchin (19), and three (DHC1, DHC2, and DHC3) (21) in HeLa cells. Conventional cytoplasmic dynein heavy chain (CyDn), DHC1A, and DHC1 are homologs and members of cytoplasmic dynein families; DLP4, DHC1B, and DHC2 are also homologs with each other. DHC2 is localized predominantly in the Golgi apparatus, whereas DHC3 is associated with as yet unidentified structures that may represent transport intermediates (21). Moreover, microinjection of antibodies to DHC2 causes dispersion of the Golgi complex (21). A recent study of cytoplasmic dynein gene knockout mice demonstrated that in $CyDn^{-/-}$ cells, the Golgi complex is fragmented and widely distributed in the cytoplasm, and late endosomes are distributed to the periphery of the cells (67) (Fig. 3). Thus, it is likely that both CyDn and DHC2 are involved in the formation and distribution of the Golgi complex. Because we do not have any data indicating the interaction of specific isoforms of the dynactin complex with specific members of the cytoplasmic dynein family, loss-of-function experiments with respect to specific members of the cytoplasmic dynein family need to be carried out to understand their individual roles in organelle transport.

Regulation of Bidirectional Transport

Regulation of bidirectional transport is an important mechanism in organelle transport. Because immunocytochemical analysis of ligated nerves indicates that the retrograde motor cytoplasmic dynein is conveyed to the cell periphery by anterogradely transported cargoes, a mechanism is required to dissociate anterograde and retrograde motors from membranous organelles at the cell periphery and to reassociate retrograde motors with retrogradely transported cargoes (59) (Fig. 3). The dissociation of anterograde and retrograde motors may involve the phosphorylation of kinesin and cytoplasmic dynein to regulate bidirectional transport (68). The activation and inactivation of motor proteins by phosphorylation and dephosphorylation may also be involved (68). It is also noteworthy that the direction of vesicle movement is regulated by the presence or absence of a tightly bound plus-end kinesin motor; that is, vesicles move retrogradely only when a retrograde motor is bound to the vesicles in the absence of an anterograde motor (69). In addition, the observation that guanosine 5′-O-(3′-triotriphosphate) (GTP-γ-S) inhibits vesicle transport in isolated squid axoplasm (70) suggests that small G proteins could be involved in the regulation of the association of motors to cargoes or the activation of the motors. More studies are necessary to clarify this issue.

Targeting of Motors

As outlined above, certain members of the kinesin and dynein superfamilies have been discovered, and each member has its own cargoes, although some redundancy may exist. Both kinesin and cytoplasmic dynein exhibit saturation binding to the vesicles, and proteolysis of vesicle membrane proteins abolishes binding (71). The COOH-terminal domain of KHC is thought to bind membranous cargoes, and this binding could be modulated by KLCs (32, 70, 72). However, it is currently unknown how a motor recognizes its own cargo.

As a candidate receptor for kinesin, kinectin was purified by kinesin affinity column chromatography (73). However, because kinectin also binds to cytoplasmic dynein and because it is plausible that cytoplasmic dynein binds to the cargo membranes through a dynactin-actin binding protein network (73), kinectin may not be a binding protein for kinesin in the cargo membranes, but may be a regulator of the binding of kinesin to the cargo. The identification of receptors for KIFs on cargoes is an important topic for future research. Cytoplasmic dynein may bind indirectly to cargoes through an interaction among the 74-kD intermediate chain, $p150^{Glued}$, dynamitin, and Arp1-actin short filament (Fig. 5). Because the Arp1-actin filament resembles the actin short filament in the spectrin-actin network underneath the erythrocyte plasma membrane, and because specific isoforms of ankyrin and spectrin associated with Golgi complex have been identified and because dynactin interacts with spectrin (74), it is possible that a receptor protein in the cargo membrane binds to the Arp1-actin filament and cytoplasmic dynein through the ankyrin-spectrin network (Fig. 5).

However, there are at least two or three members of the cytoplasmic dynein family that seem to bind to different organelles (20, 21). Although nothing is known about the isoforms of dynactin associating with new members of the cytoplasmic dynein family (because cytoplasmic dynein seems to be linked to its cargo indirectly through dynactin), the specificity of binding of cytoplasmic dynein with the cargo should be determined by the interaction of dynactin and receptors on the cargo. Although we have some understanding of how the motor binds to its own cargo, further studies are required to answer this intriguing question.

Conclusions and Future Challenges

A cell uses a surprisingly large number of microtubule-associated motor proteins (more than 20 KIFs and at least two or three

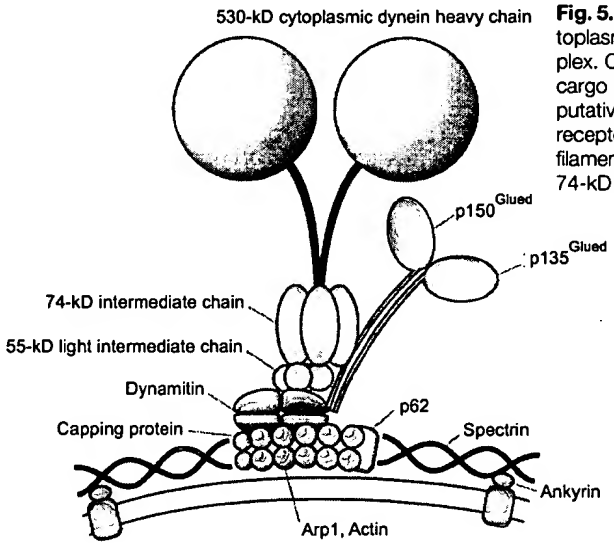

Fig. 5. Schematic representation of cytoplasmic dynein and dynactin complex. Cytoplasmic dynein is linked with cargo membranes indirectly through a putative interaction among membrane receptor, ankyrin, spectrin, Arp1-actin filament, dynamitin, $p150^{Glued}$, and the 74-kD intermediate chain.

cytoplasmic dynein family members have been identified), which precisely control the direction and velocity of transport of various kinds of cargoes. These cargoes include distinct types of membranous organelles, possibly protein complexes and mRNA (*1*, *43–45*, *75*, *76*). The control of transport involves developmental regulation as well. Because important motors still remain to be identified, we need to search for them. Molecular cell biological and molecular genetics approaches may enable the characterization of newly identified members, the analyses of the functions of each member, and the biological significance of the transports performed by each member. Understanding the mechanism of the recognition of the correct cargo by each motor is also important. How and where the cargoes bind to their correct motors, and how the cargoes are dissociated from the motors at their destination, are also intriguing topics for future research. The regulation mechanisms may involve phosphorylation and dephosphorylation of motors and receptors on the cargoes, or may involve small G proteins and signal transduction cascades. Organelle transport and membrane traffic are deeply related to each other. In the near future, we will fully understand how the cell transports and sorts proteins and lipids to their appropriate destinations in order to build itself. We are beginning to obtain abundant new information regarding the mechanism of organelle transport in cells, while at the same time fascinating new questions are arising that will lead to exciting research in this field.

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